

**MOLECULAR CHARACTERISATION OF *Flavobacterium* spp. AND
INVESTIGATION OF THEIR BIOFILM-FORMING CAPACITY IN THE
TILAPIA AQUACULTURE SYSTEM.**

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Science at the University of Stellenbosch**

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DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis is my own, unaided work. It has been submitted for the degree of Master of Science in the University of Stellenbosch. It has not been submitted previously, in its entirety or in part, at any other university.

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ABSTRACT

Fish infections caused by pathogenic *Flavobacterium* spp. are a major problem in the aquaculture industry worldwide, often leading to large economic losses. Thirty-two *Flavobacterium* spp. isolates, obtained from various diseased fish species and biofilm growth, were characterised genetically using 16S rRNA gene sequencing, 16S rRNA gene PCR restriction fragment length polymorphism (RFLP), randomly amplified polymorphic DNA (RAPD) PCR, repetitive extragenic palindromic (REP) element PCR, plasmid profiling, whole cell protein (WCP) and outer membrane protein (OMP) analyses. The biofilm-forming capability of five genetically heterogeneous *Flavobacterium* spp. study isolates was investigated using a modified microtiter-plate adherence assay, as well as flow cell studies. Experimental infection studies with Mozambique tilapia (*Oreochromis mossambicus*) were carried out in order to determine the virulence of the *Flavobacterium* spp. study isolates. 16S rRNA gene sequence analysis showed the *Flavobacterium* spp. study isolates were closely related, and 97% sequence similarity was shared with published *F. johnsoniae* sequences. A high degree of genetic heterogeneity was displayed by the *Flavobacterium* spp. study isolates following RAPD-PCR, REP-PCR and OMP analysis, however, based on the results obtained by plasmid profiling and WCP analysis, the isolates appeared genetically very homogeneous. The biofilm phenotype was displayed by all five *Flavobacterium* spp. isolates tested and varied from weakly to strongly adherent. No specific correlation was observed between the RAPD, REP and/or OMP profiles and degree of adherence displayed by *Flavobacterium* spp. isolates. However, a specific WCP profile (profile B), exhibited by 48% of the *Flavobacterium* spp. isolates, was linked to strong adherence. Experimental infection studies showed that *Flavobacterium* spp. isolates displayed variable levels of virulence, which could not be linked to biofilm formation, nor specific genotypes. This is the first reported isolation and characterisation of *Flavobacterium* spp. isolated from diseased fish in Southern Africa, and there appears to be significant diversity amongst the isolates which is not geographically linked nor host related.

OPSOMMING

Visinfeksies veroorsaak deur *Flavobacterium* spp. is problematies in die akwakultuur industrie wêreldwyd en lei tot groot ekonomiese verliese. Twee en dertig *Flavobacterium* spp. isolate, geïsoleer vanaf verskeie geïnfekteerde visspesies en biofilm groei, was geneties gekarakteriseer met behulp van 16S rRNS geenvolgorde, 16S rRNS geen PKR restriksie fragment lengte polimorfisme (RFLP), toevallig geamplifiseerde polimorfiese DNS (TGPD) PKR, herhaalde ekstrageniese palindromiese (HEP) element PKR, plasmied profilering, heersel protein (HSP) en buite membraan protein (BMP) analise. Die vermoë van vyf geneties heterogene *Flavobacterium* spp. isolate om biofilms te vorm was ondersoek met behulp van 'n gemodifiseerde mikrotiterplaat vashegtings toets asook vloeisel studies. Eksperimentele infeksie studies was uitgevoer op bloukurpers (*Oreochromis mossambicus*) om die virulensie van die *Flavobacterium* spp. studie isolate te toets. 16S rRNS geenvolgorde analise het getoon dat die *Flavobacterium* spp. studie isolate naby verwant was, en het 97% ooreenstemming getoon met gepubliseerde *F. johnsoniae* volgordes. TGPD-PKR, HEP-PKR en BMP analise het 'n hoë graad van heterogeniteit tussen die *Flavobacterium* spp. studie isolate aangetoon, egter, op grond van plasmied profilering en HSP analise, was die studie isolate geneties baie homogeen. Die biofilm fenotipe was getoon deur al die getoetsde *Flavobacterium* spp. isolate en het gevarieer van swak tot sterk vashegting. Geen spesifieke korrelasie was waargeneem tussen die TGPD, HEP en/of BMP profiele en graad van vashegting vertoon deur *Flavobacterium* spp. isolate nie, maar 'n spesifieke HSP profiel (profiel B), getoon deur 48% van die *Flavobacterium* spp. isolate, was verbind met sterk vashegting. Eksperimentele infeksie studies het getoon dat *Flavobacterium* spp. isolate varierende grade van virulensie vertoon het en wat met biofilm formasie of spesifieke genotipes geassosieer kon word nie. Hierdie is die eerste gedokumenteerde isolasie en karakterisering van *Flavobacterium* spp. geïsoleer van geïnfekteerde vis in Suider Afrika, en daar is beduidende diversiteit tussen die isolate wat nie geografies of gasheer geassosieerd is nie.

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LIST OF FIGURES

FIG. 2.1.	Characteristic orange/yellow spreading colonies formed on EAOA by <i>Flavobacterium</i> spp. isolates.....	51
FIG. 2.2.	Stereomicroscope image of <i>Flavobacterium</i> spp. isolate YO19 displaying the smooth colony type (40× magnification).....	52
FIG. 2.3.	Stereomicroscope image of <i>Flavobacterium</i> spp. isolate YO10 displaying the hazy colony type (40× magnification).....	52
FIG. 2.4.	Isolates presenting the hazy colony type (B) displayed superior gliding motility in comparison to isolates with the smooth colony type (A)....	53
FIG. 2.5.	Light microscope image of <i>Flavobacterium</i> spp. isolate YO10 grown in EAOB displaying Gram-negative bacilli phenotype (1000× magnification)...	55
Fig. 2.6.	Light microscope image of <i>Flavobacterium</i> spp. isolate YO20 grown on EAOA displaying Gram-negative pleomorphic phenotype (1000× magnification).....	55
FIG. 2.7.	Liberation of flexirubin pigment from <i>Flavobacterium</i> spp. cells following exposure to 10 N NaOH (A), in comparison to cells not exposed to 10 N NaOH (B).....	56
FIG. 2.8.	<i>Flavobacterium</i> spp. colonies appeared red following uptake of Congo red after growth on Congo red EAOA plates.....	56
FIG. 3.1.	Agarose gel electrophoresis of 16S rRNA gene PCR products of <i>Flavobacterium</i> spp. isolates DNA amplified with primer sets 16S-F + 16S-R, and F1 + R5. Lanes 1 and 2: isolates YO10 and YO19 amplified with primers 16S-F and 16S-R, respectively; lanes 3 and 4: isolates YO10 and YO19 amplified with primers F1 + R5, respectively, and lane 5: molecular weight marker VI [Roche, Germany (2176/1766/1230/1033/653/517/453/394/298/234-220/154bp)].....	74
FIG. 3.2.	16S rRNA gene sequence homology tree showing 2 clusters of nine selected <i>Flavobacterium</i> spp. study isolates YO10, YO11, YO15 + YO46 and YO19, YO34, YO64, YO51 + YO60, and FJOHNSONIAE AB078043 (Welker <i>et al.</i> , 2005), FRAINBOWTROUT AY374109 (Huber <i>et al.</i> , 2004), and FWUBA46 AF336355 (Bohnert <i>et al.</i> , 2001).....	74
FIG. 3.3.	16S rRNA gene sequence homology tree showing relatedness of nine selected <i>Flavobacterium</i> spp. study isolates to other related bacteria in the genus <i>Flavobacterium</i> : FAQUATILE M62797 (Woese <i>et al.</i> , 1990), FBRANCHIOPHILUM D14017 (Nakagawa and Yamasato, 1996), FC ATCC49512 AY635167 (Darwish and Ismaiel, 2005), FHYDATIS AY753068 (Welker <i>et al.</i> , 2005), Fjohnsoniae1 AY635167 (Darwish and Ismaiel, 2005), Fjohnsoniae4 M59053 (Ghera and Woese, 1992), Fjohnsoniae5 AY753067 (Welker <i>et al.</i> , 2005), FJOHNSONIAE AB078043 (Nakagawa <i>et al.</i> , 2002),	

FPSYCHROPHILUM AB07860 (Nakagawa *et al.*, 2002), FRAINBOWTROUT AY374109 (Huber *et al.*, 2004), and FWUBA46 AF336355 (Bohnert *et al.*, 2001), based on comparison of 16S rRNA gene sequences..... 76

FIG. 3.4. Agarose gel electrophoresis of 16S rRNA PCR products of *Flavobacterium* spp. isolates and various type species DNA with the *F. psychrophilum* species-specific primer set FP1 + FP2. Lane 1: O'GeneRuler 100 bp DNA ladder [Fermentas, Canada (3000/2000/1500/1200/1031/900/800/700/600/500/400/300/200/100 bp)]; lanes 2-19: isolates YO10, YO19, YO51, YO60, YO64, *E. coli* strain DH5 α , *Aeromonas* spp. isolate, *A. hydrophila*, *A. salmonicida*, *A. sobria*, *C. balustinum* NCTC 11212, *C. gleum* NCTC 11432, *C. indologenes* NCTC 10796, *C. indoltheticum* ATCC 27950, *C. joostei* LMG 18212, *C. meningosepticum* NCTC 10016, *M. odoratus* NCTC 11036, and *S. multivorum* NCTC 11343, respectively..... 77

FIG. 3.5. Agarose gel electrophoresis of 16S rRNA PCR products of *Flavobacterium* spp. isolates and various type species DNA with the *F. columnare*/*F. johnsoniae* primer set FvP F1 + FvP R1. Lanes 1-10: isolates YO10, YO19, YO51, YO60, YO64, *E. coli* strain DH5 α , *Aeromonas* spp. isolate, *A. hydrophila*, *A. salmonicida*, *A. sobria*; lanes 12-17: *C. balustinum* NCTC 11212, *C. gleum* NCTC 11432, *C. indologenes* NCTC 10796, *C. indoltheticum* ATCC 27950, *C. joostei* LMG 18212, and *C. meningosepticum* NCTC 10016, respectively, and lane 11: O'GeneRuler 100 bp DNA ladder [Fermentas, Canada (3000/2000/1500/1200/1031/900/800/700/600/500/400/300/200/100 bp)]..... 78

FIG. 3.6. Agarose gel electrophoresis of restriction fragments following digestion of the 1193 bp 16S rRNA gene amplicon with *AluI*. Lanes 1 and 19: molecular weight marker VI [Roche, Germany (2176/1766/1230/1033/653/517/453/394/298/234-220/154 bp)]; lanes 2-18: isolates YO10, YO49, YO50, YO51, YO52, YO53, YO54, YO55, YO56, YO57, YO58, YO59, YO60, YO61, YO62, YO63 and YO64, respectively..... 79

FIG. 3.7. Non-denaturing polyacrylamide gel electrophoresis of restriction fragments following digestion of the 1193 bp and 1500 bp 16S rRNA amplicons, respectively, with *HaeIII*. Lane 1: O'GeneRuler 100 bp DNA ladder [Fermentas, Canada (3000/2000/1500/1200/1031/900/800/700/600/500/400/300/200/100 bp)]; lanes 2-6: RFLP profiles of the 1193 amplicon of isolates YO10, YO19, YO51, YO60 and YO64, respectively, and lanes 7-11: RFLP profiles of the 1500 bp amplicon of isolates YO10, YO19, YO51, YO60 and YO64, respectively..... 79

FIG. 3.8. Agarose gel electrophoresis of restriction fragments following digestion of the 1193 bp 16S rRNA amplicon with *Hinfi*. Lanes 1-8: isolates YO10, YO49, YO50, YO51, YO52, YO53, YO54, YO55, YO56, YO57, YO58, YO59, YO60,

- YO61, YO62, YO63 and YO64, respectively, and lane 9: molecular weight marker VI [Roche, Germany (2176/1766/1230/1033/653/517/453/394/298/234-220/154 bp)]..... 80
- FIG. 3.9.** Agarose gel electrophoresis of restriction fragments following digestion of the 1193 bp 16S rRNA amplicon with *CfoI*. Lanes 1 and 17: molecular weight marker VI [Roche, Germany (2176/1766/1230/1033/653/517/453/394/298/234-220/154 bp)]; lanes 2-16: isolates YO10, YO11, YO12, YO15, YO19, YO20, YO21, YO26, YO34, YO35, YO38, YO45, YO46, YO49 and YO50, respectively..... 80
- FIG. 3.10.** Agarose gel electrophoresis of restriction fragments following digestion of the 1193 bp 16S rRNA amplicon with *CfoI*. Lanes 1 and 16: molecular weight marker VI [Roche, Germany (2176/1766/1230/1033/653/517/453/394/298/234-220/154 bp)]; lanes 2-16: isolates YO51, YO52, YO53, YO54, YO55, YO56, YO57, YO58, YO59, YO60, YO61, YO62, YO63 and YO64, respectively..... 81
- FIG. 3.11.** Agarose gel electrophoresis of restriction fragments following digestion of the 1193 bp 16S rRNA amplicon with *MspI*. Lanes 1 and 17: molecular weight marker VI [Roche, Germany (2176/1766/1230/1033/653/517/453/394/298/234-220/154 bp)]; lanes 2-16: isolates YO10, YO51, YO52, YO53, YO54, YO55, YO56, YO57, YO58, YO59, YO60, YO61, YO62, YO63 and YO64, respectively..... 81
- FIG. 3.12.** Agarose gel electrophoresis of restriction fragments following digestion of the 1193 bp 16S rRNA amplicon with *TaqI*. Lanes 1 and 19: molecular weight marker VI [Roche, Germany (2176/1766/1230/1033/653/517/453/394/298/234-220/154 bp)]; lanes 2-18: isolates YO10, YO49, YO50, YO51, YO52, YO53, YO54, YO55, YO56, YO57, YO58, YO59, YO60, YO61, YO62, YO63 and YO64, respectively..... 82
- FIG. 3.13.** Agarose gel electrophoresis of RAPD amplification products (UBC 214 primer) of *Flavobacterium* spp. isolates. Lanes 1, 7, 13, and 19: molecular weight marker VI [Roche, Germany (2176/1766/1230/1033/653/517/453/394/298/234-220/154 bp)]; lanes 2-6: isolates YO10, YO11, YO12, YO15 and YO19, respectively; lanes 8-12: isolates YO20, YO21, YO26, YO34 and YO35, respectively, and lanes 14-18: isolates YO38, YO45, YO46, YO49 and YO50, respectively..... 83
- FIG. 3.14.** Agarose gel electrophoresis of RAPD amplification products (UBC 214 primer) of *Flavobacterium* spp. isolates. Lanes 1, 7, 13, and 19: molecular weight marker VI [Roche, Germany (2176/1766/1230/1033/653/517/453/394/298/234-220/154 bp)]; lanes 2-6: isolates YO10, YO51, YO52, YO53 and YO54, respectively; lanes 8-12: isolates YO55, YO56, YO57, YO58 and

- YO59, and lanes 14-18: isolates YO60, YO61, YO62, YO63 and YO64, respectively..... 84
- FIG. 3.15.** Agarose gel electrophoresis of RAPD amplification products (UBC 214 primer) of *Flavobacterium* spp. isolates. Lanes 1, 6, and 11: O'GeneRuler 100 bp DNA ladder [Fermentas, Canada (3000/2000/1500/1200/1031/900/800/700/600/500/400/300/200/100 bp)]; lanes 2-5: isolates YO10 (repeat amplification), YO12 (repeat amplification), YO19 (repeat amplification), and YO12 (re-isolated genomic DNA), respectively, and lanes 7-10: isolates YO19 (re-isolated genomic DNA), YO65, YO66, and YO67, respectively..... 84
- FIG. 3.16.** Dendrogram of the cluster analysis of RAPD profiles of *Flavobacterium* spp. isolates following DNA amplification using random primer UBC 214. 87
- FIG. 3.17.** Agarose gel electrophoresis of REP-PCR fragments of *Flavobacterium* spp. isolates. Lanes 1, 8, and 15: O'GeneRuler 100 bp DNA ladder [Fermentas, Canada (3000/2000/1500/1200/1031/900/800/700/600/500/400/300/200/100 bp)]; lanes 2-7: isolates YO10, YO11, YO12, YO15, YO19, and YO20, respectively, and lanes 9-14: isolates YO21, YO26, YO34, YO35, YO38 and YO45, respectively..... 89
- FIG. 3.18.** Agarose gel electrophoresis of REP-PCR fragments of *Flavobacterium* spp. isolates. Lanes 1, 8, 15 and 21: O'GeneRuler 100 bp DNA ladder [Fermentas, Canada (3000/2000/1500/1200/1031/900/800/700/600/500/400/300/200/100 bp)]; Lanes 2-7: isolates YO46, YO49, YO50, YO51, YO52 and YO53, respectively, lanes 9-14: isolates YO54, YO55, YO56, YO57, YO58 and YO59, respectively, and lanes 16-20: isolates YO60, YO61, YO62, YO63 and YO64, respectively..... 89
- FIG. 3.19.** Agarose gel electrophoresis of REP-PCR fragments of *Flavobacterium* spp. isolates. Lanes 1 and 9: O'RangeRuler 200 bp DNA ladder [Fermentas, Canada (3000/2800/2600/2400/2200/2000/1800/1600/1400/1200/1000/800/600/400/200 bp)], and lanes 2-8: YO10 (repeat amplification), YO12 (re-isolated genomic DNA), YO19 (re-isolated genomic DNA), YO65, YO66, and YO67, respectively..... 90
- FIG. 3.20.** Dendrogram of the cluster analysis of REP profiles of *Flavobacterium* spp. isolates following DNA amplification using the REP primer set REP1 and REP2..... 92
- FIG. 3.21.** Agarose gel electrophoresis of plasmids from *Flavobacterium* spp. isolates. Lane1: pUCBM21 (3 kb), and lanes 2-5: isolates YO10, YO45, and YO49, respectively..... 93
- FIG. 3.22.** Electrophoretogram displaying whole cell protein profiles of *Flavobacterium* spp. isolates. Lanes 1 and 10: PageRuler™ prestained protein ladder (170/130/100/70/55/45/35/25/15/10 kDa) (Fermentas, Canada), and lanes 2-

	9: isolates YO12, YO15, YO19, YO20, YO21, YO26, YO34 and YO35, respectively.....	95
FIG. 3.23.	Electrophoretogram displaying whole cell protein profiles of <i>Flavobacterium</i> spp. isolates. Lanes 1 and 10: PageRuler™ prestained protein ladder (170/130/100/70/55/45/35/25/15/10 kDa) (Fermentas, Canada), and lanes 2-9: isolates YO38, YO45, YO49, YO50, YO51, YO52, YO53 and YO54, respectively.....	95
FIG. 3.24.	Electrophoretogram displaying whole cell protein profiles of <i>Flavobacterium</i> spp. isolates. Lanes 1 and 10: PageRuler™ prestained protein ladder (170/130/100/70/55/45/35/25/15/10 kDa) (Fermentas, Canada), and lanes 2-9: isolates YO55, YO56, YO57, YO58, YO59, YO60, YO61 and YO62, respectively.....	96
FIG. 3.25.	Electrophoretogram displaying whole cell protein profiles of <i>Flavobacterium</i> spp. isolates. Lane 1: PageRuler™ prestained protein ladder (170/130/100/70/55/45/35/25/15/10 kDa) (Fermentas, Canada); and lanes 2-6: isolates YO63, YO64, YO65, YO66 and YO67, respectively.....	96
FIG. 3.26.	Dendrogram generated by cluster analysis of whole cell protein profiles of the <i>Flavobacterium</i> spp. isolates.....	97
FIG. 3.27.	Electrophoretogram displaying outer membrane protein profiles of <i>Flavobacterium</i> spp. isolates. Lanes 1 and 10: PageRuler™ prestained protein ladder (170/130/100/70/55/45/35/25/15/10 kDa) (Fermentas, Canada), and lanes 2-9: isolates YO12, YO15, YO19, YO20, YO21, YO26, YO34 and YO35, respectively.....	100
FIG. 3.28.	Electrophoretogram displaying outer membrane protein profiles of <i>Flavobacterium</i> spp. isolates. Lanes 1 and 10: PageRuler™ prestained protein ladder (170/130/100/70/55/45/35/25/15/10 kDa) (Fermentas, Canada), and lanes 2-9: isolates YO38, YO45, YO49, YO50, YO51, YO52, YO53 and YO54, respectively.....	100
FIG. 3.29.	Electrophoretogram displaying outer membrane protein profiles of <i>Flavobacterium</i> spp. isolates. Lanes 1 and 10: PageRuler™ prestained protein ladder (170/130/100/70/55/45/35/25/15/10 kDa) (Fermentas, Canada), and lanes 2-9: isolates YO55, YO56, YO57, YO58, YO59, YO60, YO61 and YO62, respectively.....	101
FIG. 3.30.	Electrophoretogram displaying outer membrane protein profiles of <i>Flavobacterium</i> spp. isolates. Lanes 1 and 8: PageRuler™ prestained protein ladder (170/130/100/70/55/45/35/25/15/10 kDa) (Fermentas, Canada), and lanes 2-7: isolates YO63, YO64, YO65, YO66, and YO67, respectively.....	101
FIG. 3.31.	Dendrogram generated by cluster analysis of outer membrane protein profiles of <i>Flavobacterium</i> spp. isolates.....	103

FIG. 4.1.	Room temperature microtiter-plate adherence assay, where different shades of bound crystal violet display different levels of adherence, i.e., darker blue showing stronger adherence, by <i>Flavobacterium</i> spp. isolates prior to the addition of acetic acid.....	128
FIG. 4.2.	Room temperature microtiter-plate adherence assay, where different shades of bound crystal violet display different levels of adherence, i.e., darker blue showing stronger adherence, by <i>Flavobacterium</i> spp. isolates following the addition of acetic acid.....	128
FIG. 4.3.	Light microscope image depicting cells of isolate YO10 associated with the glass slide surface following 24 h flow cell incubation (1000× magnification).....	130
FIG. 4.4.	Light microscope image of isolate YO10, where pillar-shaped micro-colonies with filaments extending into the flowing medium were observed in flow cell chambers following 48 h flow cell incubation (1000× magnification).....	131
FIG. 4.5.	Light microscope image depicting mono-layered biofilm formation by isolate YO19, including polar-attached cells (arrow), following 24 h flow cell incubation (1000× magnification).....	131
FIG. 4.6.	Light microscope image depicting multi-layered biofilm formation by isolate YO19 following 48 h flow cell incubation (1000× magnification).....	132
FIG. 4.7	Light microscope image depicting the irregular surface of a biofilm formed by isolate YO19 with pillar-like structures (arrow) protruding into the flowing medium following 48 h flow cell incubation (1000× magnification)..	132
FIG. 4.8.	Light microscope image depicting micro-colony formation by isolate YO19 at an increased flow rate of 6 rpm following 24 h flow cell incubation (1000× magnification).....	133
FIG. 4.9.	Light microscope image depicting increased micro-colony formation by isolate YO19 at a flow rate of 6 rpm following 48 h flow cell incubation. Long filaments (arrow) extending into flowing media can be seen (1000× magnification).....	133
FIG. 4.10.	Light microscope image depicting cells of isolate YO51 associated with the glass slide surface following 24 h flow cell incubation (1000× magnification).....	134
FIG. 4.11.	Light microscope image depicting thick multi-layered biofilm growth by isolate YO51 at the glass-perspex interface following 48 h flow cell incubation (1000× magnification).....	134
FIG. 4.12.	Light microscope image depicting cells of isolate YO60 associated with the glass surface following 24 h flow cell incubation (1000× magnification).....	135

FIG. 4.13.	Light microscope image depicting micro-colony formation by cells of isolate YO60 following 24 h flow cell incubation (1000× magnification).....	135
FIG. 4.14.	Light microscope image depicting increased amounts of glass surface-associated cells of isolate YO60 following 48 h flow cell incubation (1000× magnification).....	136
FIG. 4.15.	Light microscope image depicting glass surface-associated cells of isolate YO64 with randomly spread polar-attached cells following 24 h flow cell incubation (1000× magnification).....	136
FIG. 4.16.	Light microscope image depicting mixed mono- and multi-layered biofilm-formation by isolate YO64 with abundant polar-attached cells following 48 h flow cell incubation (1000× magnification).....	137
FIG. 4.17.	Light microscope image depicting mono-layered biofilm-formation by the <i>A. hydrophila</i> isolate following 24 h flow cell incubation. Micro-colonies (A) and multiple polar-attached cells (arrow) can be seen protruding into the flowing medium (1000× magnification).....	137
FIG. 4.18.	Light microscope image depicting multi-layered biofilm-formation by the <i>A. hydrophila</i> isolate cells following 48 h flow cell incubation (1000× magnification).....	138
FIG. 4.19.	Light microscope image depicting scattered micro-colonies formed by the <i>Vibrio</i> spp. isolate following 24 h flow cell incubation (1000× magnification).....	138
FIG. 4.20.	Light microscope image depicting complex multi-layered biofilm-formation by the <i>Vibrio</i> spp. isolate following 48 h flow cell incubation (1000× magnification).....	139
FIG. 4.21.	Light microscope image depicting cells of isolate YO19 and <i>A. hydrophila</i> randomly attached to the glass surface following 24 h flow cell incubation (1000× magnification).....	140
FIG. 4.22.	Light microscope image depicting cells of isolate YO19 and <i>A. hydrophila</i> forming complex heterogeneous microcosms following 48 h flow cell incubation (1000× magnification).....	140
FIG. 4.23.	Light microscope image depicting cells of isolate YO51 associated with the glass surface, randomly spread among attached <i>A. hydrophila</i> cells following 24 h flow cell incubation (1000× magnification).....	141
FIG. 4.24.	Light microscope image depicting multi-layered biofilm-formation by isolate YO19 (A) and micro-colonies of the <i>Vibrio</i> spp. isolate (B) on the outer surface following 48 h flow cell incubation (1000× magnification)..	141
FIG. 4.25.	Light microscope image depicting multi-layered biofilm-formation by isolate YO51 (A) and micro-colonies of the <i>Vibrio</i> spp. isolate (B) on the outer surface following 48 h flow cell incubation (1000× magnification)..	142

FIG. 4.26.	Light microscope image of an aquaculture tank water sample depicting cocci (A) and pleomorphic bacilli (B) attached to the glass surface following 24 h flow cell incubation (1000× magnification).....	143
FIG. 4.27.	Light microscope image of an aquaculture tank water sample depicting curved cells (A) and spiral cells (B) attached to the glass surface following 24 h flow cell incubation (1000× magnification).....	144
FIG. 4.28.	Light microscope image of an aquaculture tank water sample depicting heterogeneous micro-colonies formed by diverse bacterial forms including long filamentous bacterial cells following 24 h flow cell incubation (1000× magnification).....	144
FIG. 4.29.	Light microscope image of an aquaculture tank water sample depicting cells resembling that of <i>Vibrio</i> spp. (arrow) suspended among other cell types in a thick EPS layer following 48 h flow cell incubation (1000× magnification).....	145
FIG. 4.30.	Light microscope image of an aquaculture tank water sample seeded with isolate YO19 depicting biofilm formation by diverse bacterial cells suspended in a thick layer of EPS following 48 h of flow cell incubation. Long slender bacilli (arrow) resembling cells of isolate YO19 were visible among the multi-species consortia (1000× magnification).....	146
Fig. 4.31.	Light microscope image of sterile aquaculture water inoculated with isolate YO19 depicting scattered attached pleomorphic cells (arrow) following 24 h of flow cell incubation (1000× magnification).....	146
FIG. 4.32.	Light microscope image depicting encapsulated cells of <i>Flavobacterium</i> spp. isolate YO10 (1000× magnification).....	147
FIG. 5.1.	Caudal fin rot observed in <i>Oreochromis mossambicus</i> after 72 h of exposure to isolate YO60 following severe handling stress.....	160
FIG. 5.2.	Skin ulceration observed in <i>Oreochromis mossambicus</i> after 96 h of exposure to isolate YO60 following severe handling stress.....	160
FIG. 5.3.	Agarose gel electrophoresis of amplification products from <i>Flavobacterium</i> spp. DNA with the UBC 214 primer. Lanes 1, 8, and 15: O'GeneRuler 100 bp DNA ladder [Fermentas, Canada (3000/2000/1500/1200/1031/900/800/700/600/500/400/300/200/100 bp)]; Lanes 2-7: isolates YO19 (original culture), YO19 (re-isolated from infected fish), YO64 (original culture), YO64 (re-isolated from infected fish), spleen sample from tank 3 (fingerprint identical to isolate YO19), and liver sample from tank 3 (unique fingerprint), respectively, and lanes 9-14: isolates YO60 (original culture), YO60 (re-isolated from infected fish), YO51 (original culture), YO51 (re-isolated from infected fish), YO12 (original culture), and YO12 (re-isolated from infected fish), respectively.....	162

FIG. 1A.	Alignments of 16S rRNA gene sequences of <i>Flavobacterium</i> spp. study isolates YO10, YO11, YO15 + YO46 and YO19, YO34, YO64, YO51 + YO60, as well as FJOHNSONIAE AB078043 (Welker <i>et al.</i> , 2005), FRAINBOWTROUT AY374109 (Huber <i>et al.</i> , 2004), and FWUBA46 AF336355 (Bohnert <i>et al.</i> , 2001).....	191
FIG. 2A.	16S rRNA gene sequence homology tree showing relatedness of nine selected <i>Flavobacterium</i> spp. study isolates (YO10, YO11, YO15, YO19, YO34, YO46, YO51, YO60, and YO64) to other related bacteria in the genus <i>Flavobacterium</i> : FC (<i>F. columnare</i>) AU9824 AY842899, FC (<i>F. columnare</i>) ATCC49512 AY635167 (Darwish and Ismaiel, 2005), FC (<i>F. columnare</i>) GA693 AY842901, FHYDATIS AY753068 (Welker <i>et al.</i> , 2005), Fjohnsoniae5 AY753067 (Welker <i>et al.</i> , 2005), FJOHNSONIAE AB078043 (Nakagawa <i>et al.</i> , 2002), FPSYCHROPHILUM AB07860 (Nakagawa <i>et al.</i> , 1996), Fpsychrophilum2 AY662493 (Soule <i>et al.</i> , 2005), Fpsychrophilum1 AY662494 (Soule <i>et al.</i> , 2005), FRAINBOWTROUT AY374109 (Huber <i>et al.</i> , 2004), and FWUBA46 AF336355 (Bohnert <i>et al.</i> , 2001), based on comparison of 16S rRNA gene sequences.....	192

LIST OF TABLES

TABLE 2.1.	<i>Flavobacterium</i> spp. isolates, host species, geographical location and date of isolation.	50
TABLE 2.2.	Phenotypic, physiological and biochemical characterisation of <i>Flavobacterium</i> spp. isolates.	54
TABLE 3.1.	Bacterial isolates used in 16S rRNA genespecies-specific amplification.	66
TABLE 3.2.	Restriction profiles of <i>Flavobacterium</i> spp. isolates following independent cleavage of the 16S rRNA gene amplicons using restriction enzymes <i>AluI</i> , <i>CfoI</i> , <i>HaeIII</i> , <i>HinfI</i> , <i>MspI</i> , and <i>TaqI</i>	82
TABLE 3.3.	Number and sizes of RAPD-PCR products obtained from 32 <i>Flavobacterium</i> spp. isolates following amplification with the UBC 214 primer. ...	85
TABLE 3.4.	Number and sizes of REP-PCR products obtained from 32 <i>Flavobacterium</i> spp. isolates following amplification with REP1 and REP2 primers.	91
TABLE 3.5.	Whole cell protein profiles and protein molecular weight of <i>Flavobacterium</i> spp. isolates.	97
TABLE 3.6.	OMP profiles and protein molecular weights of <i>Flavobacterium</i> spp. isolates.	102
TABLE 3.7.	Discrimination indices for <i>Flavobacterium</i> spp. isolates using various typing methods.	105
TABLE 4.1.	Microtiter-plate adherence assay results of <i>Flavobacterium</i> spp. isolates.	129
TABLE 5.1.	Experimental infection of Mozambique tilapia (<i>Oreochromis mossambicus</i>) with <i>Flavobacterium</i> spp. isolates.	159

CONTENTS

DECLARATION	I
ABSTRACT.....	II
OPSOMMING	III
ACKNOWLEDGEMENTS.....	IV
LIST OF FIGURES.....	V
LIST OF TABLES.....	XIV

CHAPTER ONE INTRODUCTION AND LITERATURE REVIEW	1
1.1. TAXONOMY.....	3
1.2. HABITAT	5
1.3. PATHOGENICITY, EPIDEMIOLOGY AND VIRULENCE	6
1.3.1. <i>Flavobacterium branchiophilum</i>	7
1.3.2. <i>Flavobacterium columnare</i>	8
1.3.3. <i>Flavobacterium psychrophilum</i>	10
1.3.4. <i>Flavobacterium johnsoniae</i>	13
1.4. ISOLATION AND PRELIMINARY IDENTIFICATION.....	14
1.5. MOLECULAR IDENTIFICATION OF <i>Flavobacterium</i> spp.	19
1.5.1. 16S rRNA sequence analysis	19
1.5.2. DNA hybridization techniques.....	21
1.6. TYPING OF <i>Flavobacterium</i> spp.....	22
1.6.1. Chemotaxonomic typing methods.....	25
1.6.1.1. Whole-cell protein (WCP) analysis	26
1.6.1.2. Outer membrane protein (OMP) analysis.....	26
1.6.2. Molecular typing methods.....	28
1.6.2.1. Plasmid profiling.....	29
1.6.2.2. PCR-RFLP analysis	30
1.6.2.3. Other PCR-based typing techniques	32
1.6.2.3.1. RAPD-PCR analysis	33
1.6.2.3.2. REP-PCR analysis.....	35
1.7. BIOFILM FORMATION	36
1.7.1. Biofilms in aquatic environment	38
1.8. AIM OF THE PRESENT STUDY	40
1.8.1. Hypothesis to be tested.....	41
1.8.2. Objectives	42
1.8.3. Experimental design	42

**CHAPTER TWO PHYSIOLOGICAL AND BIOCHEMICAL CHARACTERISTICS OF
FLAVOBACTERIUM SPP. ISOLATES..... 44**

2.1.	INTRODUCTION.....	44
2.2.	MATERIALS AND METHODS	48
2.2.1.	Bacterial Isolates	48
2.2.2.	Phenotypic and Physiological Characterisation.....	48
2.2.3.	Biochemical Characterisation	50
2.3.	RESULTS.....	51
2.3.1.	Phenotypic and Physiological Characterisation.....	51
2.3.2.	Biochemical Characterisation	53
2.4.	DISCUSSION	57

**CHAPTER THREE CHEMOTAXONOMIC AND MOLECULAR CHARACTERISATION OF
FLAVOBACTERIUM SPP..... 61**

3.1	INTRODUCTION.....	61
3.2	MATERIALS AND METHODS	63
3.2.1	Genomic DNA Isolation	63
3.2.2	16S rRNA Identification Of <i>Flavobacterium</i> spp. Isolates	63
3.2.3	16S rRNA PCR-RFLP Analysis.....	66
3.2.4	RAPD-PCR Fingerprinting.....	67
3.2.5	ERIC-PCR Fingerprinting	68
3.2.6	REP-PCR Fingerprinting.....	69
3.2.7	Plasmid Profiling.....	70
3.2.8	SDS-PAGE of WCP Samples	71
3.2.9	SDS-PAGE of OMP Samples	72
3.2.10	Typeability, Reproducibility and Discrimination	73
3.3	RESULTS	74
3.3.1	16S rRNA Identification Of <i>Flavobacterium</i> spp. Isolates	74
3.3.2	16S rRNA PCR-RFLP Analysis.....	78
3.3.3	RAPD Analysis	83
3.3.4	ERIC PCR Analysis	88
3.3.5	REP-PCR Analysis.....	88
3.3.6	Plasmid Profiling Analysis	93
3.3.7	WCP Analysis.....	94
3.3.8	OMP Analysis	99
3.3.9	Typeability, reproducibility and discriminatory ability of	104
3.4	DISCUSSION	105

CHAPTER FOUR CHARACTERISATION OF THE BIOFILM-FORMING CAPABILITY OF <i>FLAVOBACTERIUM</i> SPP.	119
4.1. INTRODUCTION	119
4.2. MATERIALS AND METHODS	124
4.2.1. Microtiter adherence assays	124
4.2.2. Biofilm-forming ability in flow cell systems	125
4.2.3. Capsule stain	127
4.3. RESULTS	127
4.3.1. Microtiter-plate adherence assays	127
4.3.2. Flow cell study	129
4.3.2.1. Biofilm-formation by single, pure-culture <i>Flavobacterium</i> isolates and controls	129
4.3.2.2. Biofilm-formation of mixed cultures of bacteria	139
4.3.2.3. Biofilm-formation of bacteria in flow cell systems inoculated with aquaculture tank water	143
4.3.2.4. Biofilm-formation of isolate YO19 cells seeded into aquaculture tank water	145
4.3.3. Capsule stain analysis	147
4.4. DISCUSSION	147
 CHAPTER FIVE EXPERIMENTAL INFECTIONS WITH <i>FLAVOBACTERIUM</i> SPP. ISOLATES IN A TILAPIA AQUACULTURE SYSTEM.	 154
5.1 INTRODUCTION	154
5.2 MATERIALS AND METHODS	157
5.2.1 Experimental Infections	157
5.2.2 RAPD Fingerprinting	158
5.3 RESULTS	158
5.3.1 Experimental Infection and Virulence Analysis	158
5.3.2 RAPD-PCR Fingerprint Analysis	162
5.4 DISCUSSION	163
 CHAPTER SIX CONCLUDING REMARKS	 168
6.1 THE RESEARCH IN PERSPECTIVE	168
 REFERENCES	 171
 APPENDIX 1	 185
 APPENDIX 2	 192

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

The genus *Flavobacterium* was derived as a result of the requirement for the classification of Gram-negative, non-spore-forming, yellow pigmented rods that weakly produced acid from carbohydrate metabolism (Bernardet *et al.*, 1996). This genus rapidly acquired many poorly defined species and became a very heterogeneous group of bacteria. Aerobic, cellulolytic, gliding soil bacteria were later assigned to the genus *Cytophaga*, while soil and freshwater bacteria, phenotypically similar to *Cytophaga*, but incapable of cellulose degradation were assigned to the genus *Flexibacter* (Bernardet *et al.*, 1996). The criteria for these two genera also failed to define specific species and became very heterogeneous, containing bacteria in each respective genus that could not be sufficiently distinguished (Bernardet *et al.*, 1996). Being phenotypically similar and closely related to the genera *Cytophaga* and *Flexibacter*, the genus *Flavobacterium* was differentiated from these genera by the absence of gliding motility (Bernardet *et al.*, 1996). With the acceptance of *Flavobacterium aquatile* as the type species of the genus *Flavobacterium*, several species previously placed in this genus were reclassified and placed in new or different genera, including the genera *Bergeyella*, *Cytophaga*, *Empedobacter*, *Sphingobacterium* and *Weeksellia* (Bernardet *et al.*, 1996). Several species included in the *Flavobacterium* genus were later proposed to form the genus *Chryseobacterium* (Bernardet *et al.*, 1996). The families of these diverse genera have all been included in the phylum *Cytophaga-Flavobacterium-Bacteroides*. Extensive phylogenetic studies have shown certain families within this phylum to be only distantly related and thorough amendments have been necessary (Bernardet and Bowman, 2005).

The genus *Flavobacterium* now forms the type genus of the family *Flavobacteriaceae*, which includes genera from widely diverse ecological niches with diverse physiological characteristics. The following characteristics have

been described for *Flavobacterium* species, of which several characteristics account for general features shared among certain species classified within the family *Flavobacteriaceae* (Bernardet *et al.*, 2002). Cells are short to moderately long rods, 1-10 μm in length and 0.3-0.6 μm wide, with parallel or slightly irregular sides and rounded or slightly tapered ends. Certain *Flavobacterium* species may appear as filamentous flexible cells, depending on growth conditions. Old cultures grown on solid media may contain spherical or coccoid cells (Bernardet and Bowman, 2005; and Bernardet *et al.*, 2002). *Flavobacterium* species are Gram-negative, aerobic, chemo-organotrophic, non-spore-forming organisms that exhibit gliding motility, which is not flagellum-mediated. The optimum growth temperature differs among species and ranges from 15°C for certain psychrophilic species such as *Flavobacterium psychrophilum* (Bernardet and Bowman, 2005), to 20-30°C for most *Flavobacterium* spp. including *F. columnare* and *F. johnsoniae* (Bernardet and Bowman, 2005; and Bernardet *et al.*, 2002). Colonies are pigmented by carotenoid or flexirubin pigments, or both (Bernardet *et al.*, 1996; and Bernardet *et al.*, 2002). The major respiratory quinone is menaquinone-6 (Bernardet *et al.*, 1996; and Bernardet *et al.*, 2002). Furthermore, they are characterized by the absence of intracellular granules of poly- β -hydroxybutyrate and the absence of sphingophospholipids in the cytoplasmic membrane. The major polyamine is homospermidine (Bernardet *et al.*, 2002). Crystalline cellulose is not decomposed (Bernardet *et al.*, 2002). The DNA base composition ranges from 32-37 mol% guanine-plus-cytosine (G+C) for *Flavobacterium* spp. specifically and from 27-44 mol% G+C for the family *Flavobacteriaceae* (Bernardet *et al.*, 1996; and Bernardet *et al.*, 2002).

Flavobacteriaceae consist of mostly saprophytic species in terrestrial and aquatic environments, although several members are commonly isolated from and associated with disease in humans and animals, e.g., *Chryseobacterium indologenes*, *C. meningosepticum*, *Flavobacterium columnare*, *F. psychrophilum*, *Myroides odoratimimus*, *Ornithobacterium rhinotracheale*, *Riemerella anatipestifer*, *Tenacibaculum maritimum* and *Weeksella virosa* (Bernardet *et al.*,

1996; Bernardet *et al.*, 2002; Decostere *et al.*, 1997; Madetoja *et al.*, 2001; and Madsen and Dalsgaard, 2000). Some species are regarded as true pathogens, e.g., *F. columnare*, *F. branchiophilum* and *F. psychrophilum* (Bernardet and Bowman, 2005; Bernardet *et al.*, 1996; Bernardet *et al.*, 2002; Decostere *et al.*, 1997; Madetoja *et al.*, 2001; and Madsen and Dalsgaard, 2000). *F. columnare*, *F. psychrophilum*, *F. branchiophilum*, *F. aquatile* and *F. johnsoniae* as well as other *Cytophaga*-like bacteria cause disease in various species of fish (Bernardet and Bowman, 2005; Bernardet *et al.*, 1996; Bernardet *et al.*, 2002; Crump *et al.*, 2001; Decostere *et al.*, 1997; Madetoja *et al.*, 2001; and Madsen and Dalsgaard, 2000). These fish-pathogenic species have been widely studied due to their economic significance worldwide. Very little is known about the pathogenesis of flavobacterial fish disease and further investigation in this regard is of great importance.

1.1 TAXONOMY

Following DNA-rRNA hybridization studies and the analysis of 16S rRNA gene sequences, the genera *Flavobacterium*, *Cytophaga*, and *Flexibacter* belongs to one of 10 main phylogenetic branches of the *Bacteria* (Bernardet *et al.*, 1996). This branch is widely referred to as the *Cytophaga-Flavobacterium-Bacteroides* phylum, rRNA superfamily V, the "flavobacterium-bacteroides" phylum, or the *Flavobacterium-Cytophaga* complex (Bernardet and Bowman, 2005; Bernardet and Nagakawa, 2005; Bernardet *et al.*, 1996; and Bernardet *et al.*, 2002). The *Cytophaga-Flavobacterium-Bacteroides* phylum has undergone considerable taxonomic modifications during the last decade. In 2002, the *Cytophaga-Flavobacterium-Bacteroides* phylum consisted of the families *Bacteroidaceae*, *Flavobacteriaceae*, *Cytophagaceae*, *Sphingobacteriaceae*, and *Spirosomaceae* as well as a number of taxa unassigned to any family (Bernardet and Nagakawa, 2005; and Bernardet *et al.*, 2002). Several polar isolates have since been assigned to the new family *Cryomorphaceae* (Bernardet and

Nagakawa, 2005). *Cryomorphaceae*, along with the families *Bacteroidaceae*, *Flavobacteriaceae* and *Sphingobacteriaceae*, have been firmly defined on a phenotypic and genomic basis. However, taxa included in the families *Cytophagaceae* and *Spirosomaceae* are only distantly related (Bernardet and Nagakawa, 2005; and Bernardet *et al.*, 2002). The family *Flavobacteriaceae* has retained its position in the *Cytophaga-Flavobacterium-Bacteroides* phylum, as described by Bernardet and Nagakawa (2005), and currently includes the genera *Aequorivita*, *Arenibacter*, *Bergeyella*, *Capnocytophaga*, *Cellulophaga*, *Chryseobacterium*, *Coenonia*, *Croceibacter*, *Empedobacter*, *Flavobacterium*, "*Fucobacter*", *Gelidibacter*, *Mesonina*, *Muricauda*, *Myroides*, *Ornithobacterium*, *Polaribacter*, *Psychroflexus*, *Psychroserpens*, *Riemerella*, *Salegentibacter*, *Tenacibaculum*, *Ulvibacter*, *Vitellibacter*, *Weeksellia* and *Zobellia* (Bernardet and Nagakawa, 2005). Certain species which are unaffiliated to any genus are also included in this family and several intra-cellular symbionts of insects (*Blattabacterium* sp.) and intracellular parasites of amoebae are also closely related to this family (Bernardet and Nagakawa, 2005; and Bernardet *et al.*, 2002).

The type genus *Flavobacterium* currently comprises of the following valid species: *F. aquatile*, *F. branchiophilum*, *F. columnare*, *F. flevense*, *F. hydatidis*, *F. johnsoniae*, *F. pectinovorum*, *F. psychrophilum*, *F. saccharophilum*, *F. succinicans*, and *F. xanthum* (Bernardet and Bowman, 2005). Additionally, the invalid taxa "*Cytophaga allerginae*", "*Flexibacter aurantiacus* subsp. *excathedrus*", "*Promyxobacterium flavum*", and "*Sporocytophaga cauliformis*" also belong to the genus *Flavobacterium* (Bernardet *et al.*, 1996). Several polar isolates from Antarctica have recently been described to form part of the *Flavobacterium* genus, namely *F. degerlachei*, *F. micromati*, *F. frigidarium*, *F. frigoris*, *F. gelidilacus*, *F. gillisiae*, *F. hibernum*, and *F. tegetincola* (Bernardet and Bowman, 2005). Other psychrophilic species recently included in the genus *Flavobacterium* include *F. limicola*, *F. omnivorum*, and *F. xinjiangense* (Bernardet and Bowman, 2005).

1.2 HABITAT

A wide variety of environments are inhabited by *Flavobacterium* spp., especially aquatic environments with varying salinity, from fresh- to saltwater, excluding hypersaline environments (Bernardet and Bowman, 2005). Thirteen of the 22 valid *Flavobacterium* spp. listed in section 1.1, are psychrophilic, indicating a predilection to cool or cold, low salinity environments, including polar waterbodies, streams, rivers, lakes and muddy soils (Bernardet and Bowman, 2005). Psychrotolerant species grow at temperatures as low as 4°C, but optimal growth temperatures usually range from 20-30°C (Bernardet and Bowman, 2005). Most *Flavobacterium* spp. represent chemoheterotrophic organisms which play an important role in the mineralization of organic matter present in aquatic environments (Bernardet and Bowman, 2005). Several *Flavobacterium* spp., including *F. pectinovorum* and *F. johnsoniae*, have been isolated from moist, organic rich sediments, however, *F. johnsoniae* has also been detected in freshwater sediments and freshwater samples collected from temperate environments (Bernardet and Bowman, 2005). Other *Flavobacterium* spp. associated with temperate freshwater environments, such as groundwater, lakes, rivers and ponds, include *F. saccharophilum*, *F. flevense*, *F. limicola* and *F. xanthum* (Bernardet and Bowman, 2005). Algal mats and freshwater systems present in Antarctica form the habitat of *F. tegetincola*, *F. hibernum*, *F. xinjiangense*, *F. gelidilacus*, *F. degerlachei*, *F. micromati*, *F. frigoris* and also *F. xanthum* (Bernardet and Bowman, 2005). *F. gillisiae* and *F. frigidarium* have been isolated from polar ice and marine water systems present in Antarctica (Bernardet and Bowman, 2005). Flavobacteria, along with certain *Acinetobacter*, *Cytophaga*, *Micrococcus* and *Pseudomonas* spp. and other bacteria, also form part of the normal bacterial flora of fish, which is usually indicative of the microbial population of the surrounding aquatic environment (Bernardet and Bowman, 2005; and Inglis and Hendrie, 1993). A number of *Flavobacterium* spp. are pathogenic or regarded as opportunistic pathogens and cause disease in a wide variety of organisms, including plants, fish and humans. *F. branchiophilum*,

F. columnare, *F. hydati*s, *F. johnsoniae*, *F. psychrophilum*, and *F. succinicans* have been associated with fish disease and have also been detected in surrounding water in the presence of disease outbreaks (Bernardet and Bowman, 2005). *Flavobacterium* spp. have also been previously associated with disease in humans, but the isolated species responsible for human infection have either been reclassified into different taxa or could not be sufficiently identified. After several human cases of lung disease occurred, an unidentified species genetically closely related to certain *Flavobacterium* spp., had been isolated from an air humidification system present in a textile factory (Bernardet and Bowman, 2005). The organism producing the bacterial endotoxin found responsible for the lung disease was shown to be a *Cytophaga* spp., but further biochemical and molecular analysis confirmed a close relationship with members of the genus *Flavobacterium* (Bernardet and Bowman, 2005). Many other pathogenic associations have been attributed to certain *Flavobacterium* spp., of which most have been found to be accidental or because of opportunistic pathogenic behaviour. For instance, *F. johnsoniae* has also been detected in "soft rot" samples of plants and vegetables, indicating possible opportunistic plant pathogenicity (Bernardet and Bowman, 2005).

1.3 PATHOGENICITY, EPIDEMIOLOGY AND VIRULENCE

The wide variety of organisms infected by *Flavobacterium* spp. could be the result of opportunistic pathogenicity or accidental exposure leading to infection. This has been shown to be the case of many fish and plant infections caused by *F. johnsoniae*, as this organism is largely saprophytic, occurring in aqueous or moist soil environments (Bernardet and Bowman, 2005). *F. aquatile*, *F. hydati*s and *F. succinicans* are also occasionally isolated from diseased fish (Bernardet and Bowman, 2005; Bernardet *et al.*, 1996; and Darwish *et al.*, 2004). Several true pathogens have been described to be the etiological agents of fish disease, including *F. branchiophilum*, *F. columnare* and *F. psychrophilum*

(Bernardet and Bowman, 2005; Bernardet *et al.*, 1996; and Bernardet *et al.*, 2002). Various species of fish serve as hosts of bacterial infections caused by these pathogens and great losses of fish in aquaculture farming worldwide has led to an increasing interest in the pathogenicity of these *Flavobacterium* species.

1.3.1 *Flavobacterium branchiophilum*

The major cause of bacterial gill disease worldwide has been attributed to the pathogenicity of *F. branchiophilum* (Bernardet and Bowman, 2005). Various fish species are infected and infections in rainbow trout and other salmonids, as well as sheatfish and silver carp have been reported (Bernardet and Bowman, 2005). The organism primarily infects gills and other superficial areas of fish with the result that *F. branchiophilum* has rarely been isolated from internal organs of infected fish and has been regarded as noninvasive (Bernardet and Bowman, 2005; and Turnbull, 1993). Isolation can be difficult due to the slow growth rate and fastidious nature of *F. branchiophilum*, and colonies of this organism are easily overgrown by other bacteria (Bernardet and Bowman, 2005; and Turnbull, 1993). Colonies of isolated *F. branchiophilum* strains appear nondiffuse, with the distinct orange-yellow colouration shared by most *Flavobacterium* spp. (Bernardet and Bowman, 2005; and Turnbull, 1993). Bacterial cells appear as characteristic slender rods or filaments with the absence of gliding motility (Bernardet and Bowman, 2005; and Turnbull, 1993). The organism has been isolated from a wide variety of temperatures, from 5°C in carp infections to over 20°C in infected eels, and infection seems non-temperature dependant (Turnbull, 1993).

Bacterial gill disease is diagnosed by the lethargic and anorexic appearance of infected fish, which is accompanied by an elevated respiratory rate and flaring of the opercula (Turnbull, 1993). The production of mucus can also be elevated to an extent where visible mucus strands trail from the gills of

infected fish (Turnbull, 1993). Initial bacterial attachment has been found to occur at the tips of epithelial microridges on the tips of distal secondary lamellae (Turnbull, 1993). Glycocalyx containing sloughed material and food particles has been suggested to aid in bacterial attachment (Turnbull, 1993). Bacterial microcolonies are formed as bacterial numbers increase, leading to exfoliation and fusion of adjacent microridges (Turnbull, 1993). Extensive proliferation and fusion of the gill epithelium leads to lamellar fusion of secondary lamellae, progressing to extensive fusion and distortion of primary lamellae, trapping debris and facilitating bacterial growth in the process (Turnbull, 1993). Secondary fungal infections and/or opercular damage commonly occur at later stages of the infection (Turnbull, 1993).

The severe gill necrosis observed in bacterial gill disease has been ascribed to the production of various enzymes and hemagglutinating activities, which contribute to the virulence of this organism (Bernardet and Bowman, 2005). Several strains have been found positive for the degradation of casein, chitin and gelatin, which may also aid in the pathogenicity of *F. branchiophilum* (Turnbull, 1993).

1.3.2 *Flavobacterium columnare*

F. columnare is the causative agent of columnaris disease, which is characterized by the appearance of greyish white to yellow erosion on the body, fin rot and gill necrosis (Decostere *et al.*, 1997; and Decostere *et al.*, 1999a). Disease usually occurs when water temperatures rise above 15°C, with optimum temperatures ranging from 20-30°C (Bernardet and Bowman, 2005; and Wakabayashi, 1993). Columnaris disease has been described in many fish species and it has been suggested that all freshwater fish species are vulnerable to *F. columnare* infection, provided that conditions are favourable to the bacterium and stressful to the fish (Wakabayashi, 1993). Some of the more prominent commercially important fish species affected by *F. columnare* include

channel catfish (*Ictalurus punctatus*), common carp (*Cyprinus carpio*), goldfish (*Cyprinus auratus*), eels (*Anguilla rostrata*, *A. japonica*, *A. anguilla*), tilapia (*Oreochromis* spp.) and salmonids, such as rainbow trout (*Oncorhynchus mykiss*), brown trout (*Salmo trutta*) and brook trout (*Salvelinus fontinalis*) (Bader *et al.*, 2003; Bernardet and Bowman, 2005; and Wakabayashi, 1993). Different environmental conditions, including water temperature, water composition and oxygen deprivation, and host-related factors, such as stocking densities and stress, as well as skin or gill damage, may greatly influence the occurrence and degree of columnaris disease in fish (Bernardet and Bowman, 2005; and Wakabayashi, 1993). *F. columnare* is usually easily isolated and grown on appropriate growth media from external lesions and infected gill tissue (Bernardet and Bowman, 2005; and Wakabayashi, 1993). When columnaris disease reaches a septicemic phase, *F. columnare* may also be isolated from internal organs, such as the spleen or kidney (Bernardet and Bowman, 2005; and Wakabayashi, 1993). Colonies grown on Anacker and Ordal's agar are distinctly orange to yellow in colour, spreading with rhizoid edges and can be adherent to the agar (Bernardet and Bowman, 2005; and Wakabayashi, 1993). Bacterial cells are Gram-negative and appear morphologically filamentous or as long bacilli, which are motile by gliding motility (Bernardet and Bowman, 2005; and Wakabayashi, 1993).

Although the gills are the major sites of infection, the skin surface, mouth and fins are also frequently attacked (Bernardet and Bowman, 2005; and Wakabayashi, 1993). Infection of the gills is indicated by the presence of yellow necrotic tissue, followed by complete destruction of the gill filaments (Bernardet and Bowman, 2005). In the initial stages of skin infection, small white zones surrounded by reddish discolouration of the skin can be observed, which progresses to severe skin erosion and ulcerative and hemorrhagic necrotic lesions (Bernardet and Bowman, 2005; and Wakabayashi, 1993). A yellowish mucoid layer frequently occurs on the surface of necrotic lesions, mainly consisting of *F. columnare* cell masses (Wakabayashi, 1993). The microscopic examination of infected fish tissue usually reveals the long slender bacilli,

displaying slow gliding movements and the aggregation of cells into column-like masses along the edges of the tissue, hence the epithet "columnare" (Bernardet and Bowman, 2005; and Wakabayashi, 1993).

The production of various enzymes, including extracellular proteases and chondroitin AC lyase, by *F. columnare* may be responsible for the extensive necrotic lesions and gill necrosis observed in columnaris disease (Bernardet and Bowman, 2005). Stringer-Roth *et al.* (2002) observed a correlation between the degree of virulence and chondroitin AC lyase activity of *F. columnare* isolates. The ability of *F. columnare* to adhere to gill tissue has been demonstrated to be an important step in its pathogenesis and highly virulent strains have been found to adhere more readily to gill tissue (Decostere *et al.*, 1999a). The adherence ability of *F. columnare* has also been correlated to the hemagglutination capacity of this organism (Decostere *et al.*, 1999a). Additionally, highly virulent *F. columnare* strains have been found to produce a thicker capsule and lectin-like components of the capsule have been proposed to be responsible for bacterial attachment to gill tissue (Decostere *et al.*, 1999a).

1.3.3 *Flavobacterium psychrophilum*

F. psychrophilum is the causative agent of serious septicemic infections in fish, such as bacterial cold-water disease and rainbow trout fry syndrome (Madsen and Dalsgaard, 2000). The pathogen was first isolated in the USA and the geographical range was long thought to be restricted to the USA and Canada (Bernardet and Bowman, 2005; Michel *et al.*, 1999; and Wakabayashi, 1993). However, *F. psychrophilum* has since been isolated in Europe, Japan, Chile and Australia (Bernardet and Bowman, 2005; Michel *et al.*, 1999; and Wakabayashi, 1993). The geographical origin of this organism remains unclear, since it has been isolated and cultured from salmonid aquaculture systems across the world. The organism could have been relocated to these areas by the introduction of fish and fish eggs via the international fish trade (Bernardet and Bowman, 2005).

The possibility of *F. psychrophilum* existing in other geographical locations has also been considered, however, this pathogen could simply have passed unnoticed since appropriate culture techniques for *F. psychrophilum* have only been developed in the last two decades, thus enabling the successful isolation and identification of this organism (Bernardet and Bowman, 2005; and Michel *et al.*, 1999). *F. psychrophilum* primarily causes disease in the salmonid family, which includes charr, trout and salmon (Madsen and Dalsgaard, 2000; and Skelton, 1993). These fish species are among the best known and most valuable to man. Less severe infections caused by *F. psychrophilum* have been reported in other fish species, such as ayu, eel and cyprinids, which may well serve as reservoirs of *F. psychrophilum* for salmonid species in river systems. As the epithet "psychrophilum" indicates, *F. psychrophilum* is a psychrophilic organism, which only infects fish at low temperatures, ranging from 3-15°C (Bernardet and Bowman, 2005; and Wakabayashi, 1993). The term "bacterial cold water disease" is also derived from the association of low temperatures with disease outbreaks caused by *F. psychrophilum*, usually at temperatures below 10°C (Bernardet and Bowman, 2005; and Wakabayashi, 1993). *F. psychrophilum* is a moderately fastidious organism with special growth requirements and successful isolation of this organism is acquired on enriched Anacker and Ordal's agar (EAOA) and low incubation temperatures with an optimum temperature range between 15°C and 20°C (Bernardet and Bowman, 2005; Michel *et al.*, 1999; and Wakabayashi, 1993). Colonies usually appear yellow and spreading with irregular or rhizoid edges on EAOA (Bernardet and Bowman, 2005; and Wakabayashi, 1993). Cell morphology may vary in old cultures, but vegetative cells of *F. psychrophilum* grown in broth cultures are Gram-negative, slender bacilli, but filamentous forms may also be observed (Wakabayashi, 1993). *F. psychrophilum* is also motile by gliding motility, which may be readily detected by the spreading nature of bacterial colonies grown on EAOA (Bernardet and Bowman, 2005).

Symptoms of bacterial cold water disease caused by *F. psychrophilum* in coho salmon and rainbow trout have been described as darkening and erosion of

the skin around the adipose fin, which progresses to the caudal peduncle, leading to ulcerative lesions exposing the spinal cord (Bernardet and Bowman, 2005). This has also been referred to as peduncle disease (Bernardet and Bowman, 2005). The infection rapidly becomes septicemic and leads to high mortality rates of up to 50% in juvenile fish (Bernardet and Bowman, 2005). Internal organs mostly infected during the septicemic phase include the spleen and kidneys, which readily serve as isolation sites for *F. psychrophilum* (Bernardet and Bowman, 2005). Infection of the gills and other body parts, including the region anterior to the dorsal fin and the lower jaw, have also been reported (Bernardet and Bowman, 2005; and Wakabayashi, 1993). In turn, rainbow trout fry syndrome mainly affects rainbow trout fry and fingerlings and may lead to extremely high mortality of up to 70% (Bernardet and Bowman, 2005). The symptoms of this septicemic infection are limited and anemia, lethargy, loss of appetite, dark colouration and exophthalmia may be observed (Bernardet and Bowman, 2005). Other additional symptoms previously described include blisters and skin ulcers, as well as mouth and eye lesions in fingerlings (Bernardet and Bowman, 2005). *F. psychrophilum* may also be found present in tissue samples of internal organs, such as the liver, heart, kidneys and especially the spleen (Bernardet and Bowman, 2005).

The virulence of certain *Flavobacterium* spp. have been correlated with the ability of these organisms to degrade host tissue through the activity of extracellular enzymes (Holt *et al.*, 1993; Secades *et al.*, 2001; and Secades *et al.*, 2003). The metalloproteases, designated Fpp1 and Fpp2, present in *F. psychrophilum*, and their activity on a wide range of matrix and muscle protein substrates have indicated a contribution to colonisation and invasion of fish tissues (Secades *et al.*, 2001; and Secades *et al.*, 2003). In addition to extracellular proteases, chondroitin lyase is also produced, which may contribute to the necrotic lesions present in diseased fish (Bernardet and Bowman, 2005). Adherence of this organism to gill tissue, as with *F. columnare*, has also been demonstrated (Bernardet and Bowman, 2005; and Nematollahi *et al.*, 2003). Nematollahi *et al.* (2003) established a correlation between high virulent strains

and increased bacterial attachment to a gill perfusion model. *F. psychrophilum* has also been found able to agglutinate and hemolyze rainbow trout erythrocytes, which could contribute to the anemia displayed by infected fish (Lorenzen *et al.*, 1997). Other enzymatic activities of *F. psychrophilum*, such as the degradation of elastin, collagen, casein and gelatin, may also contribute to the pathogenicity of this organism (Bernardet and Bowman, 2005; and Holt *et al.*, 1993). However, the true mechanism of virulence in *F. psychrophilum* is still poorly understood.

1.3.4 *Flavobacterium johnsoniae*

Although regarded primarily as an opportunistic fish pathogen, *F. johnsoniae* has been implicated in several disease outbreaks in different fish species worldwide (Bernardet and Bowman, 2005). Specific conditions are necessary for *F. johnsoniae* infections to occur in farmed barramundi with disease outbreaks occurring after a sudden drop in temperature and increased water turbidity, indicating increased amounts of suspended solids (Bernardet and Bowman, 2005). The organism has been isolated from external lesions in fish, which has been described as very similar to superficial lesions caused by *F. columnare* (Bernardet and Bowman, 2005; Bernardet *et al.*, 1996; and Darwish *et al.*, 2004). *F. johnsoniae* isolates form yellow pigmented colonies and exhibit rapid gliding motility, which may be observed through microscopy (Bernardet and Bowman, 2005; Darwish *et al.*, 2004; and McBride, 2004). A high degree of genetic heterogeneity has been found in strains that have been classified as *F. johnsoniae* according to phenotypic and chemotaxonomic analysis, indicating the possibility for isolates currently listed as *F. johnsoniae* species to belong to several other, new species (Bernardet and Bowman, 2005).

1.4 ISOLATION AND PRELIMINARY IDENTIFICATION

Flavobacterium spp. are strictly aerobic, chemoheterotrophic organisms, which can be grown on several different types of organic media (Bernardet and Bowman, 2005). Many species can grow on rich media, although most *Flavobacterium* spp. inhabit temperate, nutrient-poor environments and isolation is usually more successful on low nutrient media and at lower incubation temperatures, as low as 4°C depending on the species (Bernardet and Bowman, 2005). In general, Anacker and Ordal's media in combination with an incubation temperature of 20°C is used for cultivation of most *Flavobacterium* spp. (Bernardet and Bowman, 2005). The concentration of NaCl especially affects the growth of many fish pathogenic species on rich media, as they generally inhabit freshwater environments (Bernardet and Bowman, 2005). Therefore, many studies have focused on the development and modification of appropriate media for the successful isolation of pathogenic species. The use of Anacker and Ordal's agar, also termed Cytophaga agar, has long been accepted for the isolation of pathogenic species (Bernardet and Bowman, 2005; and Wakabayashi, 1993). Several studies improved the growth of *F. psychrophilum* by modification of Anacker and Ordal's agar (Bernardet and Bowman, 2005; and Wakabayashi, 1993), including increased tryptone content or added fish blood (Crump *et al.*, 2001), enrichment with fetal bovine serum, and the addition of skimmed milk in combination with D(+) galactose, D(+) glucose and L-rhamnose (Daskalov *et al.*, 1999). Other media rendering good growth of *F. psychrophilum* include modified Shieh medium, Hsu-Shots medium and TYES agar (Crump *et al.*, 2001; Holt *et al.*, 1993; and Wakabayashi, 1993). Incubation temperatures differ among species and strains and usually depends on the temperature of the environment they have been isolated from (Bernardet and Bowman, 2005). Strains of *F. columnare* isolated from temperate and tropical environments have been found to grow at temperatures of 16°C and 37°C, respectively, but generally, as with *F. johnsoniae* and *F. hydatidis*, optimal growth temperatures range between 20-30°C (Bernardet and Bowman, 2005; and Decostere *et al.*,

1998). In turn, *F. psychrophilum* grows at low temperatures, ranging from 4-23°C, although weak growth has been documented at 25°C (Bernardet and Bowman, 2005; and Madetoja *et al.*, 2001). *F. psychrophilum* is able to enter a viable but non-cultivable state, which has complicated the investigation of this organism tremendously (Bernardet and Bowman, 2005; Madetoja *et al.*, 2003; and Michel *et al.*, 1999). Improvements in the Anacker and Ordal's agar medium used for the isolation and routine cultivation of *F. psychrophilum*, have improved the cultivation of *F. psychrophilum* to a large extent (Michel *et al.*, 1999; and Michel and Garcia, 2003). Optimum temperatures for other psychrophilic species vary from 11°C for *F. omnivorum* and *F. xinjiangense* to 26°C for *F. hibernum* and around 15-20°C for all the other species (Bernardet and Bowman, 2005). *F. branchiophilum* has been found to be the most fastidious among the fish pathogenic *Flavobacterium* spp. and the best growth is usually obtained on original Anacker and Ordal's agar at an incubation temperature of 18°C, although a temperature range of 20-25°C has also been found convenient (Bernardet and Bowman, 2005). Invalid taxa belonging to the genus *Flavobacterium*, including "*Cytophaga allerginae*", "*Flexibacter aurantiacus* subsp. *excathedrus*", "*Promyxobacterium flavum*", and "*Sporocytophaga cauliformis*" grow well on Anacker and Ordal's agar, nutrient agar and modified Shieh's agar at an incubation temperature of 25°C (Bernardet and Bowman, 2005). Colonies formed by *Flavobacterium* spp. on Anacker and Ordal's agar differ slightly in appearance, including colour varieties of light yellow, yellow to orange types, and spreading, motile strains, to non-spreading colonies with regular edges formed by non-motile strains or species such as *F. branchiophilum* (Bernardet and Bowman, 2005). Certain *Flavobacterium* spp. are not only mucoid or sticky in appearance, such as *F. pectinovorum*, but also adhere strongly to the surface of agar, as in the case of many *F. columnare* strains (Bernardet and Bowman, 2005). Pigments present in the different species may vary between carotenoid or flexirubin type pigments, responsible for the distinct yellow to orange colour of colonies, or even both, although non-pigmented strains have also been detected (Bernardet and Bowman, 2005).

Members of the genus *Flavobacterium* can readily be distinguished from species of other genera by a combination of physiological and biochemical characteristics. Bernardet *et al.* (2002) have described several characteristics to form part of minimal standards for the description of new *Flavobacterium* spp. classified within the genus *Flavobacterium*, which include the following criteria: colony morphology on Anacker and Ordal's agar; the adherence of colonies to the surface of Anacker and Ordal's agar; the adsorption of Congo red; growth at 25°C and on marine, nutrient and trypticase-soy agar; the presence of gliding motility; the production of flexirubin type pigments; the degradation of aesculin, agar, alginate, carboxymethylcellulose, casein, chitin, DNA, gelatin, pectin, starch, L-tyrosine and urea; the production of a brown diffusible pigment on L-tyrosine agar; the formation of a precipitate on egg yolk agar; β -galactosidase activity; the utilization of glucose as sole carbon and energy source; acid production from aerobic carbohydrate metabolism, tested in ammonium salt medium; the production of H₂S and cytochrome oxidase; the reduction of nitrate and the susceptibility to the vibriostatic compound O/129 (Bernardet *et al.*, 2002).

Flavobacterium spp. cells from young broth cultures grown in Anacker and Ordal's broth are Gram-negative slender bacilli with parallel or slightly irregular sides and rounded or slightly tapered ends (Bernardet and Bowman, 2005). Cell length varies from 2 to 5 μ m, although slightly shorter cells (1 μ m) or longer flexible filamentous cells (10 to 40 μ m) have been noted under certain growth conditions (Bernardet and Bowman, 2005; and Wakabayashi, 1993). Cell width usually varies between 0.3 and 0.5 μ m (Bernardet and Bowman, 2005; and Wakabayashi, 1993). Aging liquid cultures of certain *Flavobacterium* species, including *F. columnare*, *F. succinicans*, *F. psychrophilum*, *F. johnsoniae* and *F. branchiophilum*, may contain "spheroplasts", spherical degenerative cells usually considered non-viable (Bernardet and Bowman, 2005).

Flagella and pili have not yet been detected in *Flavobacterium* spp., although non-flagellar appendages in *F. aquatile* and fimbria-like structures in *F. branchiophilum* and *F. frigidarium*, have been reported (Bernardet and Bowman, 2005). Motility is based on a unique gliding mechanism, different from the gliding

mechanisms observed in *Myxococcus xanthus* and certain cyanobacteria (Bernardet and Bowman, 2005; and McBride, 2004). Gliding motility in *Flavobacterium* species have been observed with the use of several techniques, including the spreading and gliding ability of isolates on solid media (Jooste *et al.*, 1985; and McBride and Braun, 2004) and/or the hanging drop technique (Bernardet *et al.*, 2002). Although transporters and energy generated by the proton motive force have been linked to the gliding ability of *F. johnsoniae*, the exact mechanism of gliding is still unclear (McBride, 2004).

The detection of the flexirubin type colour pigment is based on a colour change observed when bacterial cells are covered with a 20% KOH solution or streaked onto filter paper soaked in 1N NaOH (Bernardet and Bowman, 2005). If yellow colouration is due to the presence of flexirubin, a red, purple or brown colour change may be observed, which is reversible with the addition of an acidic solution (Bernardet and Bowman, 2005). No colour change is observed when the yellow pigmentation is of carotenoid origin (Bernardet and Bowman, 2005). The adsorption of Congo red by *Flavobacterium* spp. colonies have been tested by directly flooding bacterial colonies with the dye or by growing strains on agar containing the red dye (Bernardet and Bowman, 2005). Colonies of *F. columnare* on Congo red containing media appear bright red due to the Congo red staining the extracellular galactosamine glycan present in the bacterial slime (Bernardet and Bowman, 2005). The growth of *F. psychrophilum* on media containing 30 µg/ml of Congo red though, may be strongly inhibited by the dye (Crump *et al.*, 2001).

Previously, *F. psychrophilum* had been reported to be cytochrome oxidase negative, but the presence of cytochrome oxidase has been confirmed although the reaction has been described as very weak (Bernardet and Bowman, 2005; and Holt *et al.*, 1993). The presence of cytochrome oxidase in *F. columnare* and *F. psychrophilum* has been more readily detected using discs containing dimethyl-*p*-phenylene diamine oxalate (Bernardet and Bowman, 2005). It is recommended that the cytochrome oxidase test be performed on young cultures,

less than three days old, especially with *F. psychrophilum*, which yields a very weak positive reaction (Bernardet and Bowman, 2005; and Holt *et al.*, 1993).

Polysaccharides and proteins, among other biomacromolecules, are readily degraded by certain *Flavobacterium* spp. (Bernardet and Bowman, 2005). Agar, alginate, chitin, laminarin, pectin and xylan are polysaccharide components of other organisms, including plants, algae, fungi, fish or insects, which are frequently degraded and utilized by most *Flavobacterium* spp. (Bernardet and Bowman, 2005). Fish pathogenic bacteria, such as *F. columnare* and *F. psychrophilum*, which have previously been regarded incapable of polysaccharide degradation are able to degrade complex acidic polysaccharides, which form part of connective tissue, such as chondroitin sulfate and hyaluronic acid (Bernardet and Bowman, 2005; and Holt *et al.*, 1993). Proteases produced by fish pathogenic species are able to degrade elastin, gelatin, fibronectin, fibrinogen, type IV collagen, laminin, actin and myosin (Bernardet and Bowman, 2005). These proteases, capable of degrading muscle, cartilage and connective tissue components, in combination with the ability to degrade chondroitin sulfate and hyaluronic acid may be responsible for the severe tissue necrosis displayed in fish infections. They are considered important virulence factors in fish pathogenic *Flavobacterium* spp. (Bernardet and Bowman, 2005).

Phenotypic and phylogenetic studies have revealed a high degree of homogeneity in the genus *Flavobacterium*, especially among *F. columnare* isolates (Bernardet *et al.*, 1996; Triyanto and Wakabayashi, 1999; and Urdaci *et al.*, 1998). The physiological and biochemical characteristics of *Flavobacterium* spp. used to classify newly isolated bacteria in this genus, do not necessarily allow the identification of isolates on species level. More complicated techniques, involving 16S rRNA gene sequence analysis, DNA hybridization techniques and chemotaxonomic and molecular typing, are needed for sufficient differentiation and identification of isolated *Flavobacterium* spp. at the species level. The development of efficient typing methods distinguishing members of the genus *Flavobacterium* on species and strain level have been the main focus of several studies during the last decade (Bernardet *et al.*, 1996; and Bernardet

et al., 2002). This has led to the discovery and identification of new species belonging to this genus worldwide and the recent taxonomic amendments have been largely dependent on these findings.

1.5 MOLECULAR IDENTIFICATION OF *Flavobacterium* spp.

A polyphasic approach to bacterial systematics, which integrates data obtained from phenotypic and chemotaxonomic characterisation techniques with data from genomic and phylogenetic analyses, is recommended for the identification and description of new species falling within the genus *Flavobacterium* (Bernardet *et al.*, 2002). Studies regarding the classification of newly isolated species on molecular level should include as many cultivable isolates of a possible species, which should preferably also represent a wide variety of independent sources (Bernardet *et al.*, 2002). Initially, the location of the newly isolated species within the 16S rRNA gene tree should be determined to restrict the number of possible species and the amount of technically demanding and complicated investigations, including the use of DNA hybridization techniques (Bernardet *et al.*, 2002).

1.5.1 16S rRNA gene sequence analysis

Certain bacterial species are difficult to identify using phenotypic identification schemes. Analysis of bacterial 16S rRNA gene sequence (1500 bp), due to its universal distribution among bacteria in general and the presence of species-specific regions among bacterial species, reveals the specific identity of bacterial isolates (Drancourt *et al.*, 2000). This technique also allows the determination of rRNA gene sequences from bacteria without culture, therefore enabling the study of noncultivable organisms from sample material (Bernardet and Bowman, 2005; Urdaci *et al.*, 1998; and Weisburg *et al.*, 1991). Oppong *et*

al. (2003) found this technique highly effective for tracing the identity of filamentous isolates from paper mill slimes, which revealed the presence of a possible *Flavobacterium* isolate. Several studies have extensively made use of this technique to identify and compare different *Flavobacterium* spp. at DNA level (Arias *et al.*, 2004; Baliarda *et al.*, 2002; Darwish *et al.*, 2004; Madetoja and Wiklund, 2002; Michel *et al.*, 2002; Triyanto *et al.*, 1999; Triyanto and Wakabayashi, 1999; Urdaci *et al.*, 1998; and Wiklund *et al.*, 2000). The coexistence of many *Flavobacterium* spp. in fresh water environments has made the specificity of the PCR techniques used to identify etiologic agents of fish disease belonging to this genus extremely critical (Urdaci *et al.*, 1998). Hence, the development of 16S rRNA gene species-specific primer sets for *F. columnare* (Darwish *et al.*, 2004; and Triyanto *et al.*, 1999) and *F. psychrophilum* (Toyama *et al.*, 1994; and Urdaci *et al.*, 1998), respectively, have been of great use for the rapid identification of these pathogens following disease outbreaks (Darwish *et al.*, 2004; Madetoja and Wiklund, 2002; Michel *et al.*, 2002; and Triyanto *et al.*, 1999). Nested PCR techniques involving the use of both 16S rRNA gene universal and species-specific primer sets have also been effectively used for the detection and identification of *F. psychrophilum* from water and fish tissue samples (Baliarda *et al.*, 2002; Madetoja and Wiklund, 2002; and Wiklund *et al.*, 2000). The sequences of 16S rRNA genes of all the valid *Flavobacterium* spp. and most of the recently isolated new species are available in the Genbank database according to Bernardet and Bowman (2005). However, even though the 16S rRNA gene sequencing data reveals valuable information to delineate taxa at genus and family levels, respectively, this technique should not be the only genomic method used to delineate *Flavobacterium* spp. (Bernardet and Bowman, 2005). The only exception arises with noncultivable organisms, such as certain polar species detected in ice samples (Bernardet and Bowman, 2005). Organisms sharing less than 97% 16S rRNA gene sequence relatedness rarely display more than 60% DNA homology, although exceptions exist (Bernardet *et al.*, 2002). The sequence homology values of 16S rRNA gene regions higher than 97% also do not guarantee conspecificity (Bernardet *et al.*, 2002).

Therefore, this technique should be used in combination with several other reliable genomic methods, such as DNA hybridization techniques, to delineate bacteria belonging to the genus *Flavobacterium* at the species level (Bernardet and Bowman, 2005; Bernardet *et al.*, 1996; Bernardet *et al.*, 2002; and Triyanto and Wakabayashi, 1999).

1.5.2 DNA hybridization techniques

The evaluation of DNA-DNA similarity with the use of quantitative DNA-DNA hybridization remains the acknowledged standard for delineation of bacterial species. In general, DNA relatedness among bacterial strains representing a specific species varies from approximately 70% and higher in combination with a ΔT_m value of 5°C or less (Bernardet and Bowman, 2005; Bernardet *et al.*, 1996; Bernardet *et al.*, 2002; and Triyanto and Wakabayashi, 1999). Comparative studies, with data obtained from DNA-DNA hybridization techniques, have shown the 70% cutoff value proposed for DNA similarity within *Flavobacterium* spp. to be valid (Bernardet and Bowman, 2005; Bernardet *et al.*, 1996; Bernardet *et al.*, 2002; and Triyanto and Wakabayashi, 1999). DNA relatedness values of below 70% have been obtained for certain strains among others representing the same *Flavobacterium* spp., however, reciprocal hybridization experiments or ΔT_m values below 5°C have shown that such strains regardless belong to the same species (Bernardet *et al.*, 2002). A high genomic diversity, varying from 43% to 97% in similarity, has been found among *F. columnare* strains by the use of DNA-DNA hybridization (Triyanto and Wakabayashi, 1999). Additional genomic analysis in terms of 16S rRNA gene sequencing analysis and 16S restriction fragment-length polymorphism (RFLP) analysis revealed three distinct *F. columnare* genomovars, although the phenotypic characteristics have been documented to be identical for these *F. columnare* strains (Triyanto and Wakabayashi, 1999). It has also been documented that different quantitative hybridization techniques yield different

relative binding ratios and that the comparison of results obtained by different hybridization techniques is only possible in very few cases (Bernardet *et al.*, 2002). There is a strong recommendation for testing the integrity of bacterial DNA used in genomic studies beforehand, for fragmented DNA may yield aberrant DNA relatedness values and G+C content (Bernardet *et al.*, 2002).

Extensive DNA-rRNA hybridization experiments and the determination of G+C content have been used in order to determine the phylogenetic relationships within the *Cytophaga-Flavobacterium-Bacteroides* phylum (Bernardet *et al.*, 1996). As with 16S rRNA gene sequence analysis, DNA-rRNA gene hybridization data has been found to be effective in distinguishing between several major clusters of rRNA gene branches within the rRNA superfamily V (Bernardet *et al.*, 1996). However, Bernardet *et al.* (1996) have described rRNA gene sequencing to be superior in terms of revealing deep phylogenetic relationships, compared to DNA-rRNA hybridization.

1.6 TYPING OF *Flavobacterium* spp.

Epidemiologic typing has been described as a powerful tool for the investigation of disease outbreaks caused by pathogenic bacteria in clinical settings (Maslow and Mulligan, 1996). Epidemiologic typing is commonly used to determine if small clusters of cases represent an outbreak in a specific area or to determine if recurrent infections are caused by new or relapse infections. Alternatively, it can also be used effectively to determine if outbreaks caused by certain highly resistant bacteria are the result of selective antimicrobial pressure on the microbial flora of a host, or due to the horizontal spread of a strain (Maslow and Mulligan, 1996). Epidemiologic typing can be based on phenotypic and genotypic typing methods (Maslow and Mulligan, 1996). Phenotypic typing techniques involve the detection of characteristics expressed by an organism, including biotyping, serotyping, antimicrobial susceptibility patterns, bacteriophage and bacteriocin typing, investigation of outer membrane and

whole cell proteins by SDS-PAGE, immunoblotting and multilocus enzyme electrophoresis (Maslow and Mulligan, 1996). Hence, phenotypic typing techniques involve properties subject to gene expression, which vary, based on changes in the growth conditions, growth phase and as a result of spontaneous mutations (Tenover *et al.*, 1997). Genotypic typing techniques, in turn, are based on the direct analysis of the genetic content of an organism, which is usually more stable (Maslow and Mulligan, 1996). However, the reproducibility of genotypic typing techniques can be affected by insertions or deletions of DNA into the chromosome, the gain or loss of extrachromosomal DNA, or random mutations, but is generally less subject to natural variation compared to phenotypic typing techniques (Tenover *et al.*, 1997). Genotypic typing techniques include plasmid analysis, restriction endonuclease analysis of plasmid DNA, restriction endonuclease analysis of chromosomal DNA, Southern blot analysis of chromosomal DNA, ribotyping, pulsed field gel electrophoresis (PFGE) of chromosomal DNA and typing systems using PCR (Maslow and Mulligan, 1996).

Several basic criteria have been established for evaluation of typing systems for epidemiological use. Typing systems can be characterized in terms of typeability, reproducibility, discriminatory power, practical simplicity, cost, ease of interpretation and speed (Maslow and Mulligan, 1996; and Tenover *et al.*, 1997). Typeability refers to the ability of a typing technique to give an unambiguous positive result for each given isolate, thus, giving a percentage of distinct bacterial isolates which can be assigned a positive type (Maslow and Mulligan, 1996; and Tenover *et al.*, 1997). Reproducibility refers to a typing technique's ability to produce the same result for a given isolate on repeated testing. Therefore, reproducibility is the percentage of isolates giving the same result on repeated testing. Over time, minor variation might be observed in types of isolates and, therefore, reproducibility also refers to the typed attribute's stability over time (Maslow and Mulligan, 1996; and Tenover *et al.*, 1997). Discriminatory power refers to a typing technique's ability to differentiate epidemiologically unrelated isolates (Maslow and Mulligan, 1996; and Tenover *et*

al., 1997). Ideally, the technique should assign each isolate to a different type (Tenover *et al.*, 1997). Traditional phenotypic typing methods usually show lower discriminatory power compared to molecular methods (Tenover *et al.*, 1997). The use of a single numerical index of discrimination (D), based on the probability that two unrelated isolates sampled from a test population will be placed in distinct typing groups, can be calculated using the Simpson's index of diversity (Simpson, 1949 – cited by Hunter and Gaston, 1988):

$$D = 1 - \frac{1}{N(N-1)} \sum_{j=0}^{j=s} n_j(n_j - 1)$$

where D = numerical index; N = strain/isolate number in sample; s = subtype number; n_j = strain/isolate number belonging to j th subtype. The desired discrimination index of a typing scheme is $\geq 90\%$, which is sensitive to the number of groups defined by a typing scheme and the size of the dominant group.

Both phenotypic and genotypic typing methods have been used extensively in epidemiological studies for the typing of pathogenic organisms responsible for disease outbreaks in animals in veterinary, farming and environmental settings (Arias *et al.*, 2004; Bernardet *et al.*, 1996; Chakroun *et al.*, 1997; Cloeckaert *et al.*, 2003; Coquet *et al.*, 2002b; Crump *et al.*, 2001; Figueiredo *et al.*, 2005; Madetoja *et al.*, 2001; Madsen and Dalsgaard, 2000; Michel *et al.*, 2002; Szczuka and Kaznowski, 2004; Triyanto *et al.*, 1999; and Triyanto and Wakabayashi, 1999). The wide geographic distribution, diversity in host species, strain diversity and the economic importance of certain fish pathogenic *Flavobacterium* spp. have made them the subject of epidemiological typing studies, using a variety of molecular and chemotaxonomic typing methods (Arias *et al.*, 2004; Bernardet *et al.*, 1996; Chakroun *et al.*, 1997; Crump *et al.*, 2001; Figueiredo *et al.*, 2005; Madetoja *et al.*, 2001; Madsen and Dalsgaard,

2000; Michel *et al.*, 2002; Triyanto *et al.*, 1999; and Triyanto and Wakabayashi, 1999).

1.6.1 Chemotaxonomic typing methods

Chemotaxonomic typing information is mostly acquired through the analysis of the structure and composition of the cell wall and membrane of bacteria (Bernardet and Bowman, 2005). Analysis of the fatty acid and whole-cell protein composition of certain *Flavobacterium* spp. has revealed taxonomic information at the genus and species levels (Bernardet and Bowman, 2005; Bernardet *et al.*, 1996; and Bernardet *et al.*, 2002;). Additionally, several studies have characterized antigens, including glycoproteins and low- and high-molecular-mass lipopolysaccharides (LPS), which might serve as possible targets for vaccines and diagnostic tests (Crump *et al.*, 2001; Bernardet and Bowman, 2005; MacLean *et al.*, 2001; MacLean *et al.*, 2003; Merle *et al.*, 2003; and Vinogradov *et al.*, 2003). Serological typing has also been extensively performed on certain fish pathogenic species for potential application in epidemiological studies and diagnostic tests (Bernardet and Bowman, 2005). Via bacteriocin typing, certain strains of *F. columnare* have been found to produce bacteriocins, which are lethal to different strains of the same species, and bacteriocin susceptibility patterns were detected in certain specific *F. columnare* types (Bernardet and Bowman, 2005). A degree of correlation between these bacteriocin types and serotypes has been noted (Bernardet and Bowman, 2005). Large numbers of *Flavobacterium* spp. isolates have also previously been classified using multilocus enzyme electrophoresis (Bernardet and Bowman, 2005), in addition to several commercial available identification galleries, which include biochemical tests and enzyme galleries (Bernardet and Bowman, 2005; Bernardet *et al.*, 1996; and Bernardet *et al.*, 2002).

1.6.1.1 Whole-cell protein (WCP) analysis

The use of SDS-PAGE for analyzing whole-cell protein preparations is a highly effective typing tool (Laemmli, 1970). Protein profiles obtained are differentiated based on variation in the molecular weight of proteins (band position), concentration of proteins present (band intensity), and number of different proteins (number of bands) present in a specific profile. Electrophoretograms (scans of protein band patterns or profiles) of bacterial strains have been considered unique under standardized conditions and genetically closely related bacteria usually display similar or identical protein profiles (Plikaytis *et al.*, 1986). This technique is usually used in combination with biochemical and other phenotypic data, as well as DNA relatedness experiments (Plikaytis *et al.*, 1986).

A good correlation between the high DNA homology and high similarity of whole-cell protein patterns has been observed in the family *Flavobacteriaceae*, and certain *Flavobacterium* spp. have been readily identified by typical protein profiles (Bernardet and Bowman, 2005; and Bernardet *et al.*, 2002). Bernardet *et al.* (1996) found whole-cell protein profiles of different strains of *F. columnare* and *F. psychrophilum*, respectively, to be very similar and the identification of these taxa could readily be done on the basis of SDS-PAGE data.

1.6.1.2 Outer membrane protein (OMP) analysis

The proteins present in the outer membrane of bacteria belong to one of two major classes, viz., lipoproteins, which are anchored to the outer membrane with N-terminal lipid tails, and integral proteins, which are referred to as outer membrane proteins (OMPs), containing membrane-spanning regions (Bos and Tommassen, 2004). The isolation of OMPs is a much more complex process, compared to whole-cell protein preparations, as separation from the inner membrane constituents, lipopolysaccharides and phospholipids is essential

(Benedí and Martínez- Martínez, 2001). Typing analysis of OMPs, as with WCP analysis, requires the separation of proteins according to their molecular weight by SDS-PAGE. The evaluation of OMP profiles fall under the same criteria as WCP profiles, however, relatively fewer bands are usually present in OMP profiles compared to WCP profiles.

Porins are the most well studied OMPs due to their importance in antimicrobial resistance, especially in the investigation of multiresistant clinical isolates (Benedí and Martínez- Martínez, 2001). However, porins and other OMPs of bacteria also have an important function in the recognition of specific binding sites and adherence of organisms and can reveal important information on the biofilm forming ability of bacteria (Decostere *et al.*, 1999b; Donlan, 2002; Stickler, 1999; and Wang *et al.*, 2002). A number of surface attachment-deficient mutants of *Pseudomonas fluorescens* have been found to lack a cell surface protein, designated ClpP (Stickler, 1999). ToxR, a transmembrane regulatory protein, has been found to affect the biofilm forming ability of the fish pathogen *Vibrio anguillarum* (Wang *et al.*, 2002). Additionally, ToxR has also been found to positively and negatively regulate the production of major outer membrane proteins in *Vibrio* spp. This function includes the positive regulation of a specific OMP present in *V. anguillarum* serotype O1, sharing high amino acid sequence homology with an outer membrane porin, OmpU, of *V. cholerae* (Wang *et al.*, 2002). The loss and gain of these OMPs may alter the cell surface structures or charges, enhancing the biofilm formation of mutant strains (Wang *et al.*, 2002). The adherence of *F. columnare* to gill tissue has been strongly suggested to be dependent on a cell surface lectin, defined as a glycoprotein (Decostere *et al.*, 1999b). The role of the proteins present in the outer membrane of pathogenic *Flavobacterium* spp. has not been investigated in great depth and at present have not been specifically linked to biofilm formation and investigation in this regard is of great importance.

1.6.2 Molecular typing methods

Molecular typing methods have certain advantages over chemotaxonomic typing methods, including higher discriminatory power, broader application to a diversity in bacterial species and are often less time-consuming (Tenover *et al.*, 1997). The use of molecular typing methods has been helpful in investigations regarding outbreaks caused by a wide range of bacterial pathogens, including resistant bacterial strains responsible for human disease (Tenover *et al.*, 1997). The genetic heterogeneity in the chromosomal DNA among different bacterial species allows molecular typing systems to distinguish among epidemiologically unrelated isolates (Tenover *et al.*, 1997). Methods such as PFGE, plasmid fingerprinting, arbitrarily-primed polymerase chain reaction (AP-PCR) assays and DNA restriction assays are powerful tools for analyzing the genetic variability among bacterial pathogens and are frequently used in epidemiological typing studies (Tenover *et al.*, 1997). However, pathogenic organisms frequently associated with infections are mostly a subset of a large number of strains that constitute a species (Tenover *et al.*, 1997). This subset may exhibit a rather high genetic homogeneity, which complicates the differentiation of such organisms with the use of molecular typing techniques (Tenover *et al.*, 1997). Hence, a polyphasic approach, making use of a combination of these techniques, along with phenotypic typing methods is still recommended in epidemiological typing studies.

Epidemiological typing studies of *Flavobacterium* spp. have included the use of several molecular typing techniques for the differentiation and identification of different flavobacterial pathogens associated with fish disease. The use of plasmid profiling, ribotyping, RFLP analysis of chromosomal DNA and PCR products, random amplified polymorphic DNA (RAPD) PCR, and DNA hybridization techniques have been effectively used in combination with phenotypic typing techniques for the typing of these organisms (Arias *et al.*, 2004; Bernardet and Bowman, 2005; Bernardet *et al.*, 1996; Chakraborty *et al.*, 1997; Crump *et al.*, 2001; Izumi *et al.*, 2003; Lorenzen *et al.*, 1997; Madsen and

Dalsgaard, 2000; Michel *et al.*, 2002; Thomas-Jinu and Goodwin, 2004; Triyanto *et al.*, 1999; and Triyanto and Wakabayashi, 1999).

1.6.2.1 Plasmid profiling

Plasmids are extrachromosomal DNA elements, which are present in most clinically or environmentally important bacterial isolates (Tenover *et al.*, 1997). Plasmid profiling involves investigating the number and sizes of the plasmids present, which is then used as a basis for strain identification (Maslow and Mulligan, 1996; and Tenover *et al.*, 1997). This technique has been successfully used for the analysis of both nosocomial infection outbreaks and community-acquired infection outbreaks (Tenover *et al.*, 1997). However, this technique discriminates poorly among strains or isolates containing few or no plasmids (Maslow and Mulligan, 1996). The reproducibility of this technique can also be poor due to the existence of supercoiled or circular plasmids in molecular forms that migrate differently during gel electrophoresis (Maslow and Mulligan, 1996). Additionally, plasmids are mobile genetic elements and may be lost or gained by bacterial cells over time (Maslow and Mulligan, 1996).

Plasmid fingerprints of *F. psychrophilum* have revealed a number of plasmids present in this species (Bernardet and Bowman, 2005). Five different plasmid profiles have previously been found among mainly Danish *F. psychrophilum* isolates and a 3.2 kb plasmid has commonly been associated with strains isolated from disease outbreaks in Europe (Bernardet and Bowman, 2005; and Lorenzen *et al.*, 1997). A similar study performed by Madsen and Dalsgaard (2000) found a 3.3 kb plasmid present in the majority of Danish *F. psychrophilum* isolates studied. Plasmid profiles also revealed the presence of a 50 kb plasmid in four of the isolates and a few additional plasmids, ranging from 2.2 to 5.5 kb in size, spread randomly among isolates (Madsen and Dalsgaard, 2000). No clear correlation between virulence and 3.3 kb plasmid content could be established in this study, as most of the isolates, both virulent and non-

virulent, harbored the same 3.3 kb plasmid (Madsen and Dalsgaard, 2000). Other studies performed on *F. psychrophilum* isolates have found up to eleven different plasmid profiles and revealed that most isolates contained at least a single plasmid (Bernardet and Bowman, 2005). Additionally, a large number of *F. psychrophilum* strains, with a diverse geographical distribution, have been found to harbor the same 3.5 kb plasmid (Bernardet and Bowman, 2005). Bernardet and Bowman (2005) have proposed that these reported plasmids, ranging from 3.2 to 3.5 kb, could be identical due to the rough methods used for the evaluation of plasmid size. This same 3.5 kb plasmid, designated pCP1, has been used to create cloning vectors for the molecular manipulation of *F. johnsoniae* to aid in the identification of genes involved in the gliding motility machinery (McBride, 2004; and McBride and Kempf, 1996). The plasmid content of other pathogenic *Flavobacterium* spp. has not yet been investigated and further studies may be needed to identify plasmids, investigate plasmid fingerprints and establish a possible correlation between plasmid content and virulence (if any) of these organisms.

1.6.2.2 PCR-RFLP analysis

Conventional chromosomal restriction endonuclease analysis patterns consist of many bands and can be very difficult and time-consuming to interpret (Maslow and Mulligan, 1996). PCR-based restriction endonuclease analysis is a much more rapid technique and, depending on the strains and amplification products digested, this technique can have good discriminatory power and reproducibility. Sufficiently large enough PCR products, ranging from 1000 to 2000 bp, are ideal for further analysis with restriction endonuclease digestion (Riley, 2004). Frequently-cutting enzymes are usually used to digest PCR products, resulting in smaller fragments, which are then resolved by agarose or acrylamide gel electrophoresis, depending on the expected DNA fragment sizes (Riley, 2004). DNA sequences targeted by PCR containing a certain degree of

heterogeneity among the strains tested will generate distinct band patterns upon endonuclease digestion and gel electrophoresis (Riley, 2004). When the targeted DNA sequence involves rRNA gene sequences, the distinct epithet "amplified rDNA restriction analysis" (ARDRA) is used (Riley, 2004). However, due to the conserved nature of rRNA gene within a genus, discriminatory power of this technique is mostly limited to the species level of most microorganisms (Riley, 2004).

PCR-RFLP analysis of the 16S rRNA gene of *F. columnare* has been widely used in molecular characterisation studies of this fish pathogen (Arias *et al.*, 2004; Bernardet and Bowman, 2005; Michel *et al.*, 2002; Triyanto *et al.*, 1999; and Triyanto and Wakabayashi, 1999). Triyanto and Wakabayashi (1999) have found three unique *F. columnare* 16S rRNA gene RFLP patterns, with the use of a 16S rRNA gene universal PCR primer set and *HaeIII* digestion. Additionally, the 16S rRNA gene RFLP patterns each corresponded to three different DNA-DNA hybridization groups and have indicated genomic diversity among *F. columnare* strains (Triyanto and Wakabayashi, 1999). However, these *F. columnare* strains have been shown to be phenotypically identical (Triyanto and Wakabayashi, 1999). Consequently, *F. columnare* strains have been classified in three genomovars, based on 16S rRNA gene RFLP analysis (Triyanto and Wakabayashi, 1999). RFLP analysis performed on *F. columnare* strains by Arias *et al.* (2004) and Michel *et al.* (2002), using the same 16S rRNA gene universal primer set and subsequent *HaeIII* digestion, have confirmed the three genomovars previously proposed by Triyanto and Wakabayashi (1999). The RFLP results obtained through restriction endonuclease digestion of 16S rRNA gene amplicons of *F. columnare* with the use of *HinfI*, *HhaI*, *RsaI* and *MspI*, have been reported to show poor discriminatory power and are not recommended (Triyanto and Wakabayashi, 1999). Hence, the choice of restriction endonucleases in RFLP analysis is important.

Recently, *F. psychrophilum* has been successfully classified into four different genotypes using RFLP analysis of the gyrase subunit B gene amplicon obtained by amplification using both universal and species-specific primer sets

(Izumi *et al.*, 2000). Although the different amplicons generated by the two different primer sets resulted in different RFLP profiles, a certain degree of correlation has been established between RFLP patterns and species of fish host the *F. psychrophilum* strains have been isolated from (Bernardet and Bowman, 2005; and Izumi *et al.*, 2003). PCR-RFLP analysis of genes from *Flavobacterium* spp. has thus been described as a rapid and effective method for genotyping and differentiating among species and strains related to this genus and can potentially be used for fish-disease control of pathogenic species (Izumi *et al.*, 2003).

1.6.2.3 Other PCR-based typing techniques

Polymerase chain reaction makes use of single primers or primer sets to amplify species-specific regions or unrelated sequences selected arbitrarily or randomly (Maslow and Mulligan, 1996). This rapid technique can produce a large quantity of product from very little template DNA and may be used to detect and examine uncultivable organisms (Maslow and Mulligan, 1996). However, PCR may also amplify small amounts of contaminating DNA rendering inaccurate results (Maslow and Mulligan, 1996).

The PCR-based typing techniques have been successfully used in epidemiological typing studies and PCR results can readily be compared with results obtained from other typing methods. Frequently used PCR typing techniques include enterobacterial repetitive intergenic consensus (ERIC) sequence PCR, repetitive extragenic palindromic (REP) element PCR, RAPD-PCR and intergenic spacer region (ISR) PCR (Figueirido *et al.*, 2005; He *et al.*, 1994; Hsueh *et al.*, 1996; and Szczuka and Kaznowski, 2004). The reproducibility of these techniques may be variable, depending on the thermocycler used, the primers used and stability of DNA used as template, and may limit the use of PCR for typing of certain organisms and epidemiological

investigations of certain pathogens (He *et al.*, 1994; and Maslow and Mulligan, 1996).

Several of these PCR techniques, such as ISR PCR (Figueirido *et al.*, 2005), RAPD-PCR (Chakroun *et al.*, 1997; and Thomas-Jinu and Goodwin, 2004), and REP-PCR have been used for the characterisation of the *Flavobacterium* spp., *F. columnare* and *F. psychrophilum* (Bernardet and Bowman, 2005).

1.6.2.3.1 RAPD-PCR analysis

The RAPD-PCR technique, also referred to as arbitrarily primed (AP) PCR, makes use of short single primers, typically 10 base pairs in size, which are not directed at any specific sequence in the bacterial genome (Power, 1996; and Tenover *et al.*, 1997). Primers hybridize at multiple random chromosomal locations where they initiate DNA synthesis at low annealing temperatures. The proximity, number and locations of the priming sites vary between strains, which will produce different DNA fingerprints in the RAPD profiles obtained from different strains after electrophoresis (Power, 1996; and Tenover *et al.*, 1997). RAPD-PCR is more sensitive to variation in temperature compared to normal PCR due to less stringent annealing temperatures and short primers used. The use of different thermocyclers, causing greater temperature variation, could lead to inconsistent results and greatly affects the reproducibility of this technique (He *et al.*, 1994). Additionally, the concentration of template DNA as well as the concentration of the primer used, will affect the PCR products amplified and therefore, the reproducibility, if changed (Power, 1996). It is advised to test a number of different primers on a number of strains under standard laboratory conditions in order to obtain the most suitable primer or primer sets for the species investigated (Power, 1996). There is also the risk that amplification of contaminating DNA may take place (Riley, 2004). Therefore, this technique should be applied to DNA templates isolated from organisms grown in pure

culture (Riley, 2004). Overall, RAPD typing remains a comparative typing technique and can be used in epidemiological typing investigations, which may contribute to the control of disease outbreaks in both hospital and environmental settings (Power, 1996).

There are two ways of interpreting RAPD results, visual examination and/or use of computer software (Power, 1996). RAPD fingerprints are visually analyzed for differences in band number, band intensities and the presence or absence of bands. These variables are termed polymorphisms, which are subject to DNA insertions or deletions, presence or absence of priming sites, differences in copy number of amplicons, or varying degrees of mismatch of primer annealing between target sites. Inter-gel comparisons are easier with the use of software, especially in screening large numbers of fingerprints (Power, 1996). However, standard guidelines for interpretation of RAPD-PCR fingerprinting are not available.

Multiple *Aeromonas* spp. isolated from both clinical and environmental settings have been examined with the use of RAPD typing as well as ERIC-PCR and REP-PCR (Szczuka and Kaznowski, 2004). RAPD profiles have been found to be reproducible with only specific primers and an excellent correlation was obtained between the results of RAPD- and ERIC-PCR analysis (Szczuka and Kaznowski, 2004). Chakroun *et al.* (1997) have found certain RAPD primers produced reproducible results and allowed differentiation of several *Flavobacterium* species. A good correlation was also established between the RAPD profiles and fish host species from which the *Flavobacterium* spp. isolates had been obtained (Chakroun *et al.*, 1997). Additionally, a specific primer yielded unique RAPD profiles for all the *F. psychrophilum* strains tested (Chakroun *et al.*, 1997). Crump *et al.* (2001), similarly, found RAPD-PCR fingerprinting to be an effective method for differentiating *F. psychrophilum* from other closely related fish pathogens. Similarly, intraspecific variation among *F. columnare* isolates was demonstrated by the use of RAPD fingerprinting (Thomas-Jinu and Goodwin, 2004). The RAPD technique has been found to be highly reproducible and a correlation has been established between the RAPD

groupings of *F. columnare* and fish species of origin, possibly indicating fish species-specific virulence of *F. columnare* strains (Thomas-Jinu and Goodwin, 2004). No correlation was established between RAPD fingerprints and virulence levels of pathogenic *Flavobacterium* spp. and this requires further investigation (Chakroun *et al.*, 1997; Crump *et al.*, 2001; and Thomas-Jinu and Goodwin, 2004).

1.6.2.3.2 REP-PCR analysis

Repetitive DNA sequence elements are present in the chromosomes of both prokaryotes and eukaryotes (Riley, 2004). Many PCR-based typing assays have been developed for analysis of a variety of repetitive DNA elements identified in bacterial pathogens (Riley, 2004). REP elements, present at many sites on the chromosome of several eubacterial members, are one of the earliest reported repetitive DNA elements targeted by PCR (Power, 1996). REP elements are defined as 38-bp consensus sequences derived from REP-like elements in *Escherichia coli* and *Salmonella enterica* serotype Typhimurium (Riley, 2004). ERIC sequences are similar 126-bp elements, containing highly conserved central inverted repeat sequences, which are randomly dispersed in extragenic regions throughout the chromosome (Riley, 2004). The exact function of these repetitive DNA sequence elements is not known (Riley, 2004).

REP-PCR primers are designed to anneal near the ends of target sequences, in the outward direction, and the DNA synthesized will extend away from these target sequences (Riley, 2004). Therefore, closely situated target sequences, in the range of a few thousand bases, will act as priming sites to amplify the DNA region between them (Power, 1996). Variable numbers and sites of these repeat sequences occur in different strains of bacteria (Power, 1996). Therefore, REP-PCR of different strains will generate interrepeat fragments, varying in number and molecular weight, which will produce distinct DNA fingerprints when resolved by gel electrophoresis (Power, 1996; and Riley,

2004). The evaluation of REP fingerprints fall under the same criteria as WCP fingerprints, and can be assessed by visual analysis and/or computer software.

REP-PCR fingerprinting has been used to investigate the genomic diversity among a collection of polar *Flavobacterium* spp. isolates previously delineated by fatty acid analysis (Bernardet and Bowman, 2005). Closely related strains have been found to share the same REP profiles (Bernardet and Bowman, 2005). Additionally, strains belonging to the species *F. degerlachei* and *F. micromati*, respectively, have been found to exhibit the same REP profiles whereas strains from the species *F. frigoris* and *F. gelidilacus* exhibited variable numbers of REP profiles (Bernardet and Bowman, 2005). Hence, this technique may be used to effectively differentiate among other *Flavobacterium* spp., especially pathogenic species, or for intraspecific typing (Bernardet and Bowman, 2005).

1.7 BIOFILM FORMATION

In their natural environment, bacteria predominantly exist as sessile communities rather than free-living cells (Webb *et al.*, 2003). Bacteria attach to surfaces in aqueous environments, forming slimy coatings, containing mixed bacterial communities, which are referred to as biofilms (Bell, 2001; Stickler, 1999; and Webb *et al.*, 2003). Formation of biofilms begins with the initial attachment of discrete organisms to suitable surfaces, subsequent cell division takes place and microcolonies are formed (Bell, 2001). Biofilms may be comprised of one or more species of organisms, with the latter more frequently encountered in the natural environment, including bacteria, yeasts and protozoa (Bell, 2001). The slimy coating consists of extracellular polymeric substances (EPS), including polysaccharides and proteins, generated by the microbial cells suspended in the biofilm (Bell, 2001; and Donlan, 2002).

Analysis of biofilms and the biofilm-forming capabilities of microorganisms have suggested that OMPs and capsular polymeric substances, pili, fimbriae,

and flagella play an integral role in the ability of adherent organisms to form biofilms (Donlan, 2002; Gavín *et al.*, 2003; and Stickler, 1999). Without the above-mentioned components and impaired motility, biofilm initiation is remarkably reduced or completely lost (Donlan, 2002; Stickler, 1999; and Wang *et al.*, 2003). However, biofilm formation and the components involved therein differ in different genera and species of bacteria under different environmental conditions (Stickler, 1999).

Adherent organisms have also been found to produce intercellular signaling molecules, triggering the release of cells into the surrounding environment, as well as the aggregation of cells to build and form the biofilm (Bell, 2001). This cell signaling process, via extracellular chemicals, is referred to as quorum-sensing and it has been demonstrated in model systems of *Pseudomonas aeruginosa* where mushroom-like structures are formed with the aid of intercellular signaling molecules (Bell, 2001; and Webb *et al.*, 2003). In Gram-negative bacteria, cell signaling is achieved through the interaction of acylated homoserine lactones and cell surface receptors that control gene expression. Acylated homoserine lactones have been demonstrated to play a crucial role in the development of the biofilm specific structure and cell physiology (Stickler, 1999). Two different cell-cell signaling systems have been detected in *P. aeruginosa*, namely *lasR-lasI* and *rhlR-rhlI*, both being involved in biofilm formation (Donlan, 2002). Mutants lacking both cell signaling components form thinner, less stable biofilms, lacking the typical biofilm architecture of the wild type strain (Donlan, 2002). With the addition of homoserine lactone to the medium supplying mutant biofilms, the structure and thickness have been altered to closely resemble wild type biofilms (Donlan, 2002)

Aggregation and the formation of microcolonies by microorganisms occur under unfavourable conditions (Webb *et al.*, 2003). Several stress conditions, such as predation stress, antibiotic presence, and nutrient stress, have shown to induce microcolony formation (Webb *et al.*, 2003). Efficient microcolony formation occurs as a result of the production of specific acylated homoserine lactones (Donlan, 2002; and Webb *et al.*, 2003).

The surface area may also display important characteristics needed for bacterial adhesion. Roughness and physicochemical properties seem to play an important role in bacterial adhesion (Coquet *et al.*, 2002a; and Donlan, 2002). Rough surfaces and hydrophobic, nonpolar surfaces seem to increase the rate and extent of bacterial attachment (Donlan, 2002). However, Coquet *et al.* (2002a) have reported *Yersinia ruckeri* cells to be hydrophilic and cells are attracted to both hydrophilic and hydrophobic substances.

Microorganisms, when growing in communities such as biofilms, display remarkably different synthetic and metabolic characteristics compared to their planktonic counterparts (Bell, 2001). The survival of bacteria in their environment may depend on biofilm formation and it is important to understand the occurrence of biofilms. Biofilms are found in a variety of aqueous environments, including industrial and medical surfaces, where they constantly influence human activities (Bell, 2001; Stickler, 1999; and Webb *et al.*, 2003). Human pathogenic bacteria, forming biofilms on vascular access devices, synthetic tissue replacements, waterhandling systems in health care and sterilisation devices, pose a constant threat to human health because of their tenacious behaviour (Bell, 2001; and Stickler, 1999). Biofilm-forming capabilities of bacterial pathogens have been associated with enhanced virulence and increased resistance of biofilm bacterial cells to antimicrobial agents is constantly encountered (Coquet *et al.*, 2002a; Donlan, 2002; Stickler, 1999; and Wang *et al.*, 2003).

1.7.1 Biofilms in aquatic environment

Fish pathogens, including *Aeromonas* spp., *F. psychrophilum*, *F. columnare*, *V. anguillarum*, *V. harveyi* and *Y. ruckeri* cause tremendous problems in the aquaculture industry worldwide, and they are difficult to isolate and treat because of their fastidious nutritional growth requirements and unknown behaviour in their natural environment (Coquet *et al.*, 2002a; Darwish *et al.*,

2004; Decostere *et al.*, 1999b; Gavín *et al.*, 2003; Karunasagar *et al.*, 1996; Kondo *et al.*, 2002; and Wang *et al.*, 2003).

Organisms in aquaculture are known to form biofilms on surfaces of substances, such as wood, polyvinylchloride, metal, fibreglass and concrete, used inside tanks (Coquet *et al.*, 2002a). Biofilms present in aquaculture tanks, which continuously release organisms in the surrounding environment, and the surface colonization of materials present in fish farm tanks could be a potential source of recurrent disease for extended periods of time (Coquet *et al.*, 2002a; Coquet *et al.*, 2002b; and Leonard *et al.*, 2000). A number of studies have focussed on the biofilm-forming capabilities of aquatic pathogens, causing disease in fish (Coquet *et al.*, 2002a; Coquet *et al.*, 2002b; Decostere *et al.*, 1998; Decostere *et al.*, 1999a; Karunasagar *et al.*, 1996; Wang *et al.*, 2002; and Wang *et al.*, 2003).

Gavín *et al.* (2003) detected lateral flagellin genes in *Aeromonas* spp. and found a good correlation between the presence of the lateral flagellin gene and swarming motility, and the ability to adhere to and invade eukaryotic cells. The presence of lateral flagella has also proved to enhance the biofilm-forming capacity of *Aeromonas* spp. (Gavín *et al.*, 2003). Similarly, the biofilm-forming ability of *Y. ruckeri* has been correlated to flagellum-mediated motility (Coquet *et al.*, 2002b).

Wang *et al.* (2002) have found the *toxR* encoded ToxR transmembrane regulatory protein influenced the biofilm-forming ability of *V. anguillarum*. The ToxR protein sequence from *V. anguillarum* has also shown homology with the ToxR protein found in *V. cholerae* (Wang *et al.*, 2002). According to Wang *et al.* (2003), an OMP, OmpU, present in *V. anguillarum* may limit or prohibit biofilm formation, or may play a role in detachment from the biofilm (Wang *et al.*, 2002; and Wang *et al.*, 2003). Croxatto *et al.* (2002) have reported defective biofilm formation of *V. anguillarum* strains carrying a mutation in the *sat-vps73* DNA locus, which is related to a *V. cholerae* DNA locus carrying genes coding for biofilm formation.

The biofilm-forming abilities of *Flavobacterium* spp. have not received much attention and the role of biofilm formation by members of this genus is poorly understood. Oppong *et al.* (2003) have reported the presence of *Flavobacterium* spp. in biofilms associated with paper machine system problems. The slimy exudate produced by these organisms, along with other filamentous bacteria found in this environment, cause tremendous problems in the paper industry (Oppong *et al.*, 2003). Bremer *et al.* (2002) have evaluated the activity of chlorine on mixed bacterial biofilms comprised of *Listeria monocytogenes* and *Flavobacterium* spp. and their results indicated the *Flavobacterium* spp. tested predominated in biofilms before chlorine treatment (Bremer *et al.*, 2002).

Various studies have characterized lipopolysaccharides (LPS) and glycoproteins found in the outer membrane and capsule of *Flavobacterium* spp. (Crump *et al.*, 2001; MacLean *et al.*, 2001; MacLean *et al.* 2003; Merle *et al.*, 2003; and Vinogradov *et al.*, 2003). A correlation has been established between virulence of *F. columnare* and the presence of lectin-like carbohydrate-binding substances incorporated in the capsule of this organism (Decostere *et al.*, 1999b). Adhesion of *F. columnare* may be related to surface polysaccharide constituents (Decostere *et al.*, 1999b; and MacLean *et al.*, 2003). Furthermore, MacLean *et al.* (2003) have noted a difference in the structure of the LPS O-antigens present in *F. columnare* and *F. psychrophilum* strains. Hence, the unusual structure and composition of the outer membrane structure constituents, including glycoproteins and LPS involved in the virulence of *Flavobacterium* spp., suggest the possibility for a biofilm formation phenotype, which may in turn contribute to virulence in these organisms (Decostere *et al.*, 1999b; and MacLean *et al.*, 2003).

1.8 AIM OF THE PRESENT STUDY

Flavobacterium branchiophilum, *Flavobacterium columnare* and *Flavobacterium psychrophilum* are recognized fish pathogens (Bernardet and Bowman, 2005;

Bernardet *et al.*, 2002; Decostere *et al.*, 1998; Madetoja *et al.*, 2001; and Madsen and Dalsgaard, 2000). These rapidly spreading *Flavobacterium* spp. cause great disease problems in the freshwater aquaculture industry worldwide.

Very little work has been done on the characterisation, differentiation, biofilm formation capabilities, treatment and control of *Flavobacterium* spp. These organisms continue to cause great problems in the aquaculture industry and are extremely difficult to isolate and treat clinically (Crump *et al.*, 2001). The present study focussed on the molecular characterisation of *Flavobacterium* spp. isolates obtained from aquaculture systems, as well as investigation of their biofilm-forming capabilities. This included the identification of effective molecular methods for inter- and/or intra-strain differentiation of isolated flavobacterial species and assigning specific genotypes to isolates displaying altered virulence phenotypes. Identifying and understanding the potential mechanisms involved in biofilm formation and its correlation with virulence will assist in understanding and curbing the ability of flavobacteria to be among the major aquaculture pathogens and has potential implications for the medically important *Flavobacterium* spp.

1.8.1 Hypothesis to be tested

It was hypothesised that molecular typing methods (16S-rRNA gene PCR-RFLPs, ERIC-PCR, RAPD-PCR, REP-PCR etc.) will allow the differentiation of virulent and avirulent strains of *Flavobacterium* spp. Furthermore, it was hypothesised that virulent *Flavobacterium* spp. possess the capacity to form biofilms, facilitating their existence and transmission as pathogens within the freshwater aquaculture system.

1.8.2 Objectives

The following objectives have been established:

- a. to evaluate the use of phenotypic and molecular typing methods for inter- and intra-strain differentiation of isolated *Flavobacterium* spp.
- b. to find a possible correlation between virulence and specific genotypes of *Flavobacterium* spp.
- c. to investigate the biofilm-forming capabilities of *Flavobacterium* spp.

1.8.3 Experimental design

The following aims were pursued:

Chapter 2:

- a. to identify *Flavobacterium* spp. isolates, from a variety of aquaculture systems, according to phenotypic and biochemical characteristics.

Chapter 3:

- a. to differentiate between species/strains of *Flavobacterium* spp. isolates by 16S-rRNA gene sequence analysis; and
- b. to investigate the genetic heterogeneity of *Flavobacterium* species or strains using molecular typing methods, such as 16S-rRNA gene PCR-RFLP, RAPD-PCR, REP-PCR, ERIC-PCR, plasmid profiling, WCP and OMP analyses.

Chapter 4:

- a. to investigate the biofilm-forming capabilities of different genotypes of *Flavobacterium* species or strains using a flow-cell system and microtiter-plate adherence assays.

Chapter 5:

- a. to investigate the virulence of different genotypes of *Flavobacterium* species or strains by infection studies; and
- b. to determine the effectiveness of molecular typing for direct identification of *Flavobacterium* spp.

CHAPTER TWO

PHYSIOLOGICAL AND BIOCHEMICAL CHARACTERISTICS OF *Flavobacterium* spp. ISOLATES

2.1 INTRODUCTION

A variety of general characteristics of the genus *Flavobacterium* serve as a basis of differentiation among diverse genera included in the family *Flavobacteriaceae* (Bernardet and Bowman, 2005; Bernardet *et al.*, 1996; and Bernardet *et al.*, 2002). Phenotypic characteristics distinguishing the genus *Flavobacterium* from other genera within the family *Flavobacteriaceae* include colony morphology on EAOA (Bernardet and Bowman, 2005). *Flavobacterium* spp. grow aerobically and typically form spreading colonies, with rhizoid edges on EAOA. The characteristic gliding ability responsible for the spreading colony morphology of these cells is also better observed on low nutrient media, such as EAOA (Bernardet and Bowman, 2005). A number of newly described *Flavobacterium* spp. do not display gliding motility, including *F. branchiophilum*, which displays regular or entire edges in colony morphology indicative of the absence of gliding motility (Bernardet and Bowman, 2005). Gliding motility is strain-specific for certain species, such as *F. gelidilacus*, and gliding only occurs on nutrient-poor media (Bernardet and Bowman, 2005). Gliding motility has also been described as a characteristic highly dependent on growth conditions, such as temperature and nutrient concentration in growth medium (Bernardet *et al.*, 2002). Bacterial motility in the form of gliding motility is not restricted to the genus *Flavobacterium*, additional genera in the *Cytophaga-Flavobacterium-Bacteroides* phylum also display this form of motility along with myxobacteria and certain cyanobacteria (McBride, 2004).

The adherence ability of *Flavobacterium* spp. to the surface of solid media can also be considered as a useful identification feature (Bernardet and

Bowman, 2005). Separating *F. columnare* colonies from agar surfaces have been described as nearly impossible while colonies of *F. pectinovorum* merely exhibit sticky or mucoid consistencies (Bernardet and Bowman, 2005). *F. columnare* strains also exhibit adherence to glass surfaces if grown in broth cultures (Bernardet and Bowman, 2005). This characteristic could be an important virulence factor, aiding adherence to animal hosts in aqueous environments (Bernardet and Bowman, 2005). Subculturing may lead to a loss in adherence ability (Bernardet and Bowman, 2005).

Most isolated *Flavobacterium* spp. grown on EAOA display a characteristic orange colour. These light to bright yellow or orange pigments may belong to carotenoid or flexirubin type pigments, depending on the species (Bernardet *et al.*, 2002). Certain species may even carry both carotenoid and flexirubin type pigments (Bernardet and Bowman, 2005; and Bernardet *et al.*, 2002). The presence of flexirubin pigmentation can be evaluated by flooding a small mass of cells with 20% KOH or 1 N NaOH, which causes an immediate colour shift from yellow or orange to red, purple or brown (Bernardet and Bowman, 2005; and Bernardet *et al.*, 2002). Carotenoid type pigments are not affected by the addition of KOH or NaOH and no colour change is observed (Bernardet and Bowman, 2005).

Cell morphology of *Flavobacterium* spp. is growth condition dependent and varies from short to long filamentous cells (Bernardet and Bowman, 2005). Gram stains of bacterial cells grown in broth cultures reveal long slender Gram-negative rods or filaments. Gram stains performed on bacterial cells from old colonies grown on solid media produce Gram variable results with cells displaying remarkable morphological changes including spherical or coccoid bodies (Bernardet *et al.*, 2002). The presence of capsule has been noted in *F. columnare*, *F. hibernum* and *F. frigidarium* (Bernardet and Bowman, 2005). *F. branchiophilum* and *F. frigidarium* exhibit fimbria-like structures and nonflagellar structures have also been noticed in *F. aquatile* (Bernardet and Bowman, 2005). The presence of these structures in *Flavobacterium* spp. needs to be confirmed.

The ability of *Flavobacterium* spp. to adsorb Congo red can be tested by directly flooding colonies on solid media, or by addition of Congo red to the media, which is taken up by cells growing on the media, displaying the red dye (Bernardet and Bowman, 2005; and Crump *et al.*, 2001). In *F. columnare*, the Congo red was found to associate with extracellular galactosamine glycan in slime produced by this organism (Bernardet and Bowman, 2005).

Growth at different temperatures is useful for the differentiation of certain *Flavobacterium* spp., such as *F. columnare* and *F. psychrophilum*, and also reveals important information about the natural environment where organisms were isolated from. Although, optimal growth temperatures of *Flavobacterium* spp. range from 20-30°C and most grow well at 4°C (Bernardet and Bowman, 2005), certain *Flavobacterium* spp. have specific optimum growth temperatures. *F. psychrophilum* is a psychrophilic organism with optimum growth temperatures ranging from 15-20°C (Bernardet and Bowman, 2005), whereas *F. columnare* exhibits growth at a wider range of temperatures of up to 37°C (Decostere *et al.*, 1998). Decostere *et al.* (1998) also noted growth temperatures for *F. columnare* strains to be strain-specific and optimum growth temperatures corresponded to temperatures at isolation sites. Tropical isolates have been found to grow well at 37°C, but not at 16°C, which is the opposite for isolates from temperate environments (Decostere *et al.*, 1998).

The degradation of biomacromolecules has also been found to play an important role in the identification and differentiation process of *Flavobacterium* spp., among other genera of the family *Flavobacteriaceae* (Bernardet and Bowman, 2005). The degradation of cellulose derivatives has been found to be misleading, since a number of enzymes other than cellulases degrade these compounds. Therefore, degradation of cellulose derivatives does not demonstrate the capable *Flavobacterium* spp. to be cellulolytic (Bernardet and Bowman, 2005). A specific cellulase is required for the degradation of crystalline cellulose, filter paper, and only species capable of degrading this substance should be regarded as cellulolytic. Species belonging to the genus *Cytophaga* have been considered as cellulolytic organisms, distinguishing members of this

genus from the genus *Flavobacterium*, which are unable to undertake crystalline cellulose degradation (Bernardet and Bowman, 2005; and Bernardet *et al.*, 1996). A number of other macromolecules, including polysaccharides and proteins, are also degraded by certain *Flavobacterium* spp. strains. Polysaccharide components, including agar, alginate, chitin, laminarin, pectin and xylan, which are present in algae, plants, insects and animal hosts such as fish, are degraded by *Flavobacterium* spp. (Bernardet and Bowman, 2005; Bernardet *et al.*, 1996; and McBride, 2004). *F. columnare* and *F. psychrophilum* exhibit chondroitin sulfate and hyaluronic acid degradation, of which chondroitin sulfate degradation has been used as part of identification for *F. columnare* (Bernardet and Bowman, 2005). These acidic polysaccharides form part of connective tissue and enzymes involved in the degradation of these molecules might play an important role in the infection process and thus virulence of *Flavobacterium* spp. infecting fish (Bernardet and Bowman, 2005). A number of proteases produced by certain *Flavobacterium* spp., especially fish pathogenic species, are also considered important virulence factors (Bernardet and Bowman, 2005). Proteases produced by *Flavobacterium* spp. have been found to degrade components of muscle, cartilage and connective tissue, including actin and myosin, elastin, type IV collagen, fibrinogen, fibronectin, gelatin and laminin (Bernardet and Bowman, 2005). Protease activities of *Flavobacterium* spp. have been studied with the use of various protein assays such as skim milk-enriched agar assays (Jooste *et al.*, 1985; and Wakabayashi, 1993), gelatin-enriched agar assays (Madetoja *et al.*, 2001) and azocasein proteolytic assays (Secades *et al.*, 2001).

The use of rapid and effective methods based on physiological and biochemical characterisation is important for differentiating among new isolates. In the present study, preliminary identification and differentiation of *Flavobacterium* spp. isolates, obtained from diseased fish and biofilms present in aquaculture tanks, was accomplished using a variety of phenotypic and biochemical tests reported previously for the identification of bacteria belonging to the family *Flavobacteriaceae*.

2.2 MATERIALS AND METHODS

2.2.1 Bacterial Isolates

Thirty-two *Flavobacterium* spp. isolates (Table 2.1) were isolated from a variety of diseased fish species from various aquaculture farms situated throughout South Africa (Table 2.1). Samples from freshly netted fish displaying gill necrosis, skin ulcers and systemic disease were swabbed onto EAOA plates (Anacker and Ordal, 1955), and incubated at room temperature for 48 h. Bacterial colonies displaying a characteristic orange-yellow colour and rhizoid morphology were selected for further analysis (Bernardet *et al*, 1996). For long-term storage, stock cultures were stored in 40% (v/v) glycerol-supplemented enriched Anacker and Ordal's broth (EAOB) at -80°C.

2.2.2 Phenotypic and Physiological Characterisation

Isolates were grown on EAOA plates to study colony morphology and the presence of orange/yellow pigmentation. Growth at different temperatures was performed in triplicate on all the isolates grown overnight in EAOB at 30°C and room temperature (23°C), respectively. Motility assays were performed on modified casitone yeast (CY) agar (tryptone, 0.3 g/l; yeast extract, 1 g/l; CaCl₂.2H₂O, 1 g/l; agar, 15 g/l; and the pH adjusted to 7.2 with KOH) (Jooste *et al.*, 1985). Ten µl of overnight cultures (adjusted to a 0.5 McFarland standard) were spotted onto CY agar. The procedure was performed in triplicate for each isolate and plates were incubated at room temperature over a 5 d period. Zone diameters were taken after 5 days and the average zone size was calculated for each respective isolate. Isolates displaying the largest spreading zones were used as standard for strong gliding. Gliding ability was classified as follows: large spreading zones (distinctly > 2.5 cm) = strong gliding ability (+), small

spreading zone (distinctly < 2.5 cm) = weak gliding ability (weak +), and no spreading zone = no gliding ability (-) (Kempf and McBride, 2000).

TABLE 2.1. Isolates of *Flavobacterium* spp., host species, geographical location and date of isolation.

Strain	Species of fish (sample)	Origin	Date
YO10	Rainbow trout (<i>Oncorhynchus mykiss</i>) egg	Franschhoek, RSA	2003
YO11	Rainbow trout (<i>Oncorhynchus mykiss</i>) egg	Franschhoek, RSA	2003
YO12	Rainbow trout (<i>Oncorhynchus mykiss</i>) egg	Franschhoek, RSA	2003
YO15	Rainbow trout (<i>Oncorhynchus mykiss</i>) spleen	Franschhoek, RSA	2003
YO19	Rainbow trout (<i>Oncorhynchus mykiss</i>) spleen	Franschhoek, RSA	2003
YO20	Koi (<i>Cyprinus carpio</i>) liver	Stellenbosch, RSA	2003
YO21	Rainbow trout (<i>Oncorhynchus mykiss</i>) egg	Franschhoek, RSA	2003
YO26	Rainbow trout (<i>Oncorhynchus mykiss</i>) skin	Mpumalanga, RSA	2003
YO34	Rainbow trout (<i>Oncorhynchus mykiss</i>) skin	Mpumalanga, RSA	2003
YO35	Rainbow trout (<i>Oncorhynchus mykiss</i>) spleen	Mpumalanga, RSA	2003
YO38	Rainbow trout (<i>Oncorhynchus mykiss</i>) skin	Mpumalanga, RSA	2003
YO45	Rainbow trout (<i>Oncorhynchus mykiss</i>) spleen	Franschhoek, RSA	2003
YO46	Rainbow trout (<i>Oncorhynchus mykiss</i>) spleen	Franschhoek, RSA	2003
YO49	Rainbow trout (<i>Oncorhynchus mykiss</i>) mouth	Stellenbosch, RSA	2004
YO50	Rainbow trout (<i>Oncorhynchus mykiss</i>) fin	Stellenbosch, RSA	2004
YO51	Rainbow trout (<i>Oncorhynchus mykiss</i>) fin	Stellenbosch, RSA	2004
YO52	Koi (<i>Cyprinus carpio</i>) skin	Stellenbosch, RSA	2004
YO53	Koi (<i>Cyprinus carpio</i>) skin	Stellenbosch, RSA	2004
YO54	Koi (<i>Cyprinus carpio</i>) skin	Stellenbosch, RSA	2004
YO55	Koi (<i>Cyprinus carpio</i>) skin	Stellenbosch, RSA	2004
YO56	Koi (<i>Cyprinus carpio</i>) skin	Stellenbosch, RSA	2004
YO57	Longfin eel (<i>Anguilla mossambica</i>) gill	Stellenbosch, RSA	2004
YO58	Longfin eel (<i>Anguilla mossambica</i>) gill	Stellenbosch, RSA	2004
YO59	Longfin eel (<i>Anguilla mossambica</i>) gill	Stellenbosch, RSA	2004
YO60	Longfin eel (<i>Anguilla mossambica</i>) gill	Stellenbosch, RSA	2004
YO61	Longfin eel (<i>Anguilla mossambica</i>) gill	Stellenbosch, RSA	2004
YO62	Longfin eel (<i>Anguilla mossambica</i>) gill	Stellenbosch, RSA	2004
YO63	Longfin eel (<i>Anguilla mossambica</i>) gill	Stellenbosch, RSA	2004
YO64	Longfin eel (<i>Anguilla mossambica</i>) gill	Stellenbosch, RSA	2004
YO65	Biofilm growth (rainbow trout tank)	Franschhoek, RSA	2005
YO66	Biofilm growth (rainbow trout tank)	Franschhoek, RSA	2005
YO67	Biofilm growth (rainbow trout tank)	Franschhoek, RSA	2005

2.2.3 Biochemical Characterisation

Flavobacterium spp. isolates were characterized biochemically using the following tests: Gram stains (Hucker modification) (Gerhardt *et al.*, 1981 – cited by Jooste *et al.*, 1985) were performed on isolates grown overnight in EAOB and on EAOA. The presence of flexirubin pigments was assayed by resuspending pelleted cells, from overnight broth cultures in 10 N NaOH and 20% KOH solutions, respectively (Bernardet and Bowman, 2005). The ability of isolates to adsorb the disazo direct dye, Congo red, was tested by spotting overnight EAOB cultures onto Congo red-enriched EAOA plates (Crump *et al.*, 2001). Colonies displaying the red dye were considered a positive result.

Proteolytic activity for casein was investigated using two different protease assays. The first of these involved investigating the growth of isolates on Tryptone Yeast Extract Salts (TYES) agar (tryptone, 4 g/l; yeast extract, 0.4 g/l; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.2 g/l; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g/l, and agar, 10 g/l) enriched with 10% skim milk (Cepeda *et al.*, 2004; and Madsen and Dalsgaard, 1998). Ten μl of overnight cultures, adjusted to a 2 McFarland standard, was spotted onto skim milk-enriched TYES agar. Plates, inoculated in triplicate, were incubated at room temperature for 7 days. Subsequent clearance of the opaque medium around colonies was considered as a positive result, and zone diameters were measured after 7 days. The average zone size was calculated for each respective isolate. Isolates displaying the largest zones were used as standard for strong proteolytic activity. Proteolytic activity was classified as follows: large zone sizes (distinctly > 2.5 cm) = strong proteolytic activity (+), small zone sizes (usually distinctly ≤ 2.5 cm) = weak proteolytic activity (weak +), and no zone = no proteolytic activity (-). Additionally, isolates were also tested for protease activity using the modified azocasein protease assay described by Deane *et al.* (1987). The ability of bacterial isolates to hydrolyze gelatin was investigated using nutrient gelatin (Difco, USA) deeps. Overnight cultures (standardised to a 0.5 McFarland standard) were used to stab-inoculate gelatin deeps, in triplicate, which were incubated for 72 h at room temperature. Gelatin hydrolysis was observed by

liquefaction of the gelatin deeps, following refrigeration for 30 min to identify false-positive liquefaction (Kaminski and Ferroni, 1980).

2.3 RESULTS

2.3.1 Phenotypic and Physiological Characterisation

The results obtained during phenotypic and physiological differentiation of the *Flavobacterium* spp. isolates are listed in Table 2.2. All isolates formed bright orange spreading colonies with rhizoid edges and exhibited good growth on EAOA (Fig. 2.1).



FIG. 2.1. Characteristic orange/yellow spreading colonies formed on EAOA by *Flavobacterium* spp. isolates.

Colony morphology was described as smooth or hazy, due to the morphological appearance of colonies (Figs. 2.2-2.3) when viewed by stereomicroscopy.



FIG. 2.2. Stereomicroscope image of *Flavobacterium* spp. isolate YO19 displaying the smooth colony type (40× magnification).



FIG. 2.3. Stereomicroscope image of *Flavobacterium* spp. isolate YO10 displaying the hazy colony type (40× magnification).

Isolates exhibiting the smooth colony morphology type also adhered strongly to the EAOA plate surface (data not shown). Smooth colonies showed no or very weak gliding (Table 2.1), while majority of isolates exhibiting the hazy colony morphology type displayed strong gliding motility (Fig. 2.4). The majority of isolates showed no or weak gliding ability on modified CY agar, however, all the isolates exhibited characteristic spreading colonies on EAOA.

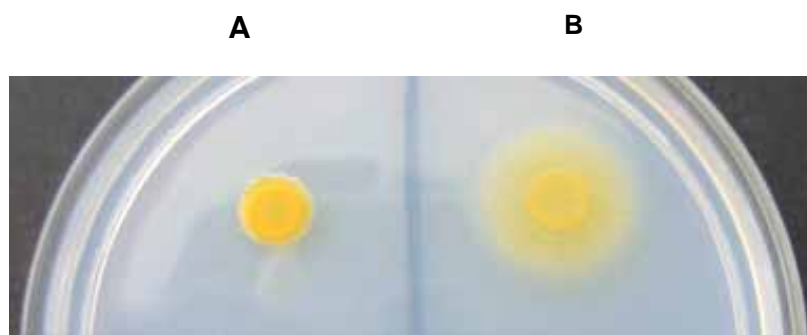


FIG. 2.4. Isolates presenting the hazy colony type (B) displayed superior gliding motility in comparison to isolates with the smooth colony type (A).

The ability of isolates to grow at room temperature was superior to growth at 30°C. During initial temperature tests, *Flavobacterium* spp. isolates showed variable growth, including no growth, at 30°C (Table 2.2). On repeated temperature tests, isolates displaying weak or no growth showed increased growth at 30°C (data not shown).

2.3.2 Biochemical Characterisation

The results obtained during biochemical differentiation of the *Flavobacterium* spp. isolates are listed in Table 2.2.

TABLE 2.2. Phenotypic, physiological and biochemical characterisation of *Flavobacterium* spp. isolates.

Isolate	Colony morphology EAOA	Gliding	Growth at different temperatures		Gram reaction		Flexirubin test		Congo red uptake EAOA	Presence of protease	
			30°C	Room temp	EAOA	EAOB	KOH (20%)	NaOH (10N)		Skim milk TYES	Gelatin
YO10	Hazy	N/A*	-	+	- bacilli‡	- bacilli	+	+	+	+	+
YO11	Hazy	N/A	-	+	- bacilli	- bacilli	+	+	+	+	+
YO12	Hazy	+	+	+	- bacilli	- bacilli	+	+	+	+	+
YO15	Hazy	+	-	+	- bacilli	- bacilli	+	+	+	+	+
YO19	Smooth	-	+	+	- bacilli	- bacilli	+	+	+	(+)	+
YO20	Smooth	-	+	+	- pleom§	- bacilli	+	+	+	(+)	+
YO21	Smooth	-	-	+	- pleom	- bacilli	+	+	+	(+)	+
YO26	Smooth	-	+	+	- pleom	- bacilli	+	+	+	(+)	+
YO34	Smooth	-	-	+	- pleom	- bacilli	+	+	+	(+)	+
YO35	Smooth	-	-	+	- pleom	- bacilli	+	+	+	(+)	+
YO38	Smooth	-	+	+	- pleom/bacilli	- bacilli	+	+	+	(+)	+
YO45	Hazy	+	+	+	- bacilli	- bacilli	+	+	+	+	+
YO46	Hazy	N/A	+	+	- pleom	- bacilli	+	+	+	+	+
YO49	Hazy	+	+	+	- bacilli	- bacilli	+	+	+	+	+
YO50	Hazy	+	+	+	- bacilli	- bacilli	+	+	+	+	+
YO51	Smooth	-	+	+	- pleom	- bacilli	+	+	+	+	+
YO52	Smooth	-	+	+	- pleom	- bacilli	+	+	+	(+)	+
YO53	Hazy	+	+	+	- bacilli	- bacilli	+	+	+	+	+
YO54	Hazy	+	+	+	- pleom/bacilli	- bacilli	+	+	+	+	+
YO55	Smooth	-	+	+	- pleom	- bacilli	+	+	+	-	+
YO56	Smooth	-	+	+	- pleom	- bacilli	+	+	+	-	+
YO57	Smooth	(+)†	(+)	+	- bacilli	- bacilli	+	+	+	(+)	+
YO58	Smooth	(+)	(+)	+	- pleom	- bacilli	+	+	+	(+)	+
YO59	Smooth	-	(+)	+	- pleom	- bacilli	+	+	+	+	+
YO60	Hazy	(+)	(+)	+	- bacilli	- bacilli	+	+	+	+	(+)
YO61	Hazy	+	(+)	+	- bacilli	- bacilli	+	+	+	+	+
YO62	Smooth	(+)	-	+	- bacilli	- bacilli	+	+	+	+	+
YO63	Smooth	-	-	+	- bacilli	- bacilli	+	+	+	(+)	+
YO64	Smooth	-	-	+	- pleom	- bacilli	+	+	+	(+)	+
YO65	Smooth	-	+	+	- bacilli	- bacilli	+	+	+	(+)	+
YO66	Smooth	-	+	+	- pleom	- bacilli	+	+	+	(+)	+
YO67	Smooth	-	+	+	- bacilli	- bacilli	+	+	+	+	+

* Cultures not available for testing (N/A)

† Weak-positive result

‡ Gram-negative bacilli

§ Gram-negative pleomorphic cells

Gram stains performed on broth cultures revealed long Gram-negative bacilli (Fig. 2.5) to filamentous organisms, whereas Gram stains performed on EAOA plate cultures varied dramatically from short Gram-negative pleomorphic cells (Fig. 2.6) to Gram-negative bacilli, respectively.



FIG. 2.5. Light microscope image of *Flavobacterium* spp. isolate YO10 grown in EAOB displaying Gram-negative bacilli phenotype (1000× magnification).

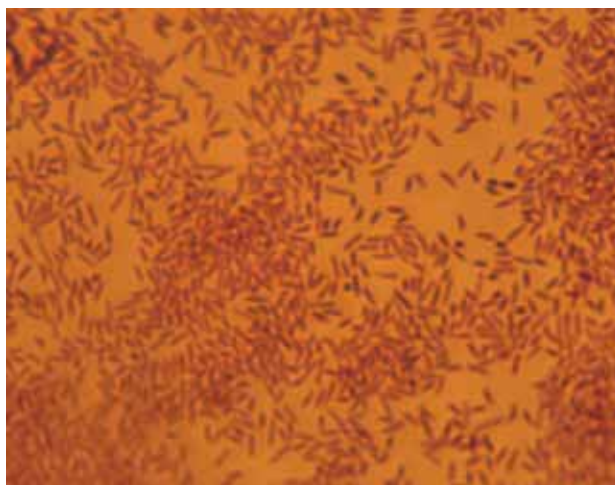


Fig. 2.6. Light microscope image of *Flavobacterium* spp. isolate YO20 grown on EAOA displaying Gram-negative pleomorphic phenotype (1000× magnification).

All the isolates tested positive for flexirubin pigment (Table 2.2 and Fig. 2.7) and the uptake of Congo red (Table 2.2 and Fig. 2.8).



FIG. 2.7. Liberation of flexirubin pigment from *Flavobacterium* spp. cells following exposure to 10 N NaOH (A), in comparison to cells not exposed to 10 N NaOH (B).

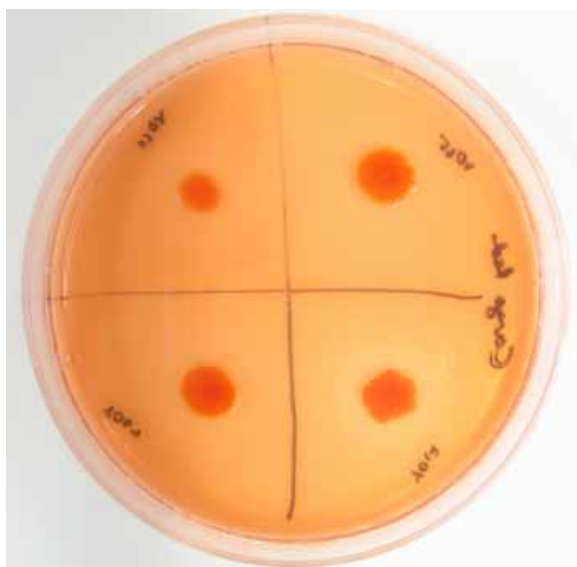


FIG. 2.8. *Flavobacterium* spp. colonies appeared red following uptake of Congo red after growth on Congo red EAOA plates.

The majority of isolates displaying each of the smooth and hazy colony morphology types exhibited weak and strong proteolytic activity for casein, respectively (Table 2.2). Only isolates YO55 and YO56 displayed no proteolytic activity for casein. Results obtained in the azocasein protease assay were not included due to inconsistent and variable results obtained during the procedure. Flexirubin pigments liberated in the supernatant appeared to affect the final optical density readings due to a chemical reaction between the flexirubin pigment and the 0.5 M NaOH that was added prior to the absorbance measurement (Deane *et al.*, 1987). All the isolates showed a positive result for gelatin hydrolysis, which took place in the top 1/4 of gelatin deeps. Only isolate YO60 displayed weak gelatin hydrolysis.

2.4 DISCUSSION

The preliminary identification of the *Flavobacterium* spp. isolates was aided by the specific growth characteristics of study isolates when grown on EAOA. The typical orange spreading colonies and presence of flexirubin pigmentation, suggested isolates were related to the genus *Flavobacterium* as described by Bernardet *et al.* (2002). The two distinct colony morphology types displayed by the *Flavobacterium* spp. isolates may be species related, as *F. columnare* strains display identical phenotypic characteristics (Bernardet and Bowman, 2005; Bernardet *et al.*, 2002; and Triyanto and Wakabayashi, 1999). However, more complex techniques, involving DNA relatedness studies, is needed to classify *Flavobacterium* spp. isolates on species level (Bernardet and Bowman, 2005; Bernardet *et al.*, 1996; and Bernardet *et al.*, 2002).

All the isolates grew well at a room temperature of 23°C, which corresponded to temperatures found at sites of isolation. Growth at 30°C was observed less frequently and seemed more strain-specific. This is in accordance with Decostere *et al.* (1998) who found differences in optimal growth temperature of *F. columnare* was strain-specific, and isolates from tropical areas displayed

higher optimal growth temperatures. Study isolates, however, displayed an adaptive nature, as isolates previously shown not to grow at 30°C showed improved growth, following serial passage (data not shown). The ability to grow at higher temperatures is also well known for *F. branchiophilum*, *F. columnare* and *F. johnsoniae* (Bernardet and Bowman, 2005; Bernardet *et al.*, 2002; and Decostere *et al.*, 1998). *F. psychrophilum* has been shown not to grow at temperatures above 25°C (Bernardet and Bowman, 2005; and Bernardet *et al.*, 2002), therefore, this eliminated study isolates being identified as *F. psychrophilum*, since they were all able to grow at 30°C (data not shown).

According to Bernardet and Bowman (2005), *Flavobacterium* spp. gliding ability is influenced by different media types and this was also observed in the present study where gliding was more apparent on EAOA plates in comparison to modified CY agar plates. Isolates displaying the smooth colony morphology type, also appeared to lack the ability to glide, indicating a possible correlation between the smooth colony morphology type and inability to glide on modified CY agar. The gliding apparatus of *Flavobacterium* spp. forms part of the outer membrane and involves membrane proteins, including lipoproteins (McBride and Braun, 2004; and McBride, 2004). Differences in the number of cell-surface polymers, such as lipoproteins, involved in *Flavobacterium* spp. gliding may alter the morphological appearance of cells. Isolates displaying the smooth colony morphology type may express lower levels of proteins and other polymers involved in the gliding machinery, displaying weaker or no gliding in comparison to isolates exhibiting the hazy morphology type.

Gram stain results obtained from broth cultures also revealed the characteristic Gram-negative filaments associated with *Flavobacterium* species (Bernardet and Bowman, 2005). Differences in cell morphology obtained from Gram stains performed on plate cultures compared to that of broth cultures were not surprising, as changes in cell wall components and morphological changes in cell structure of aging bacteria grown on solid media have been described for certain bacterial species, including *Flavobacterium* species (Bernardet and Bowman, 2005; Kondo *et al.*, 2001; and Thomas-Jinu and Goodwin, 2004).

The Congo red staining material in *F. columnare* has been shown to be an extracellular galactosamine glycan present in the slime produced by this organism (Bernardet and Bowman, 2005). Slime production may be related to the capsule present in these organisms (Decostere *et al.*, 1999a). A lectin-like substance, likely to represent a glycoprotein, present in the capsule of *F. columnare* has been shown to play an important role in bacterial attachment to gill tissue and virulence of this pathogen (Decostere *et al.*, 1999a). All the *Flavobacterium* spp. isolates tested positive for the uptake of Congo red, which may indicate the presence of capsule and possible virulence, however, further investigation is needed to confirm capsule presence.

Most of the isolates exhibited strong protease activity on both casein and gelatin substrates indicating the presence of extracellular proteases capable of casein and gelatin hydrolysis. Many proteases, including extracellular proteases, have been detected and identified in *Flavobacterium* species (Bernardet and Bowman, 2005; Secades *et al.*, 2001; and Secades *et al.*, 2003) and a correlation has been established between protease profiles and virulence of certain strains (Secades *et al.*, 2003; and Stringer-Roth *et al.*, 2002). Extracellular proteases produced by pathogenic *Flavobacterium* species have also been found to correlate with virulence and play an important role in disease symptoms of infected fish (Bernardet and Bowman, 2005; Holt *et al.*, 1993; Stringer-Roth *et al.*, 2002; and Wakabayashi, 1993). The presence of extracellular proteases responsible for protease activity, hydrolysis of casein and gelatin, demonstrated by isolates in this study could play an important role in the fish infection process and virulence of these isolates. Additionally, the majority of isolates falling within the smooth colony morphology, non- to weakly motile category showed weak or no casein hydrolysis, in contrast to hazy colony, strongly motile isolates, which displayed strong casein hydrolysis. Colony morphology, gliding ability and casein protease activity, appeared to cluster isolates within two distinct groups (Table 2.2).

Unfortunately, physiological and biochemical properties of *Flavobacterium* species do not allow identification and differentiation of newly isolated bacteria to

the species level. A more complex study, involving the use of molecular typing techniques, is needed to identify and differentiate isolated bacteria belonging to this genus on species level. For instance, the use of 16S rRNA gene sequence analysis, DNA hybridization techniques, and other molecular techniques have been described as a crucial part of identifying and differentiating members belonging to the genus *Flavobacterium* on species level (Bernardet and Bowman, 2005; and Bernardet *et al.*, 2002). Therefore, the next chapter will deal with the identification and differentiation of isolates at a molecular level, including the use of several molecular typing methods, viz: 16S rRNA gene PCR-RFLP, RAPD-PCR, REP-PCR, ERIC-PCR, WCP and OMP analyses.

CHAPTER THREE

CHEMOTAXONOMIC AND MOLECULAR CHARACTERISATION OF *Flavobacterium* spp.

3.1 INTRODUCTION

The integration of phenotypic and chemotaxonomic data with genomic and phylogenetic data is essential for the description of new species belonging to the genus *Flavobacterium* (Bernardet *et al.*, 2002). The comparison of 16S rRNA gene sequences is a powerful tool for analyzing phylogenetic and evolutionary relationships among bacteria (Weisburg *et al.*, 1991). This technique is ideal for the identification of bacterial species, which are difficult to identify using phenotypic identification schemes, as in the case of *Flavobacterium* spp. (Bernardet and Bowman, 2005; and Triyanto and Wakabayashi, 1999). The bacterial 16S rRNA gene sequence contains species-specific regions, which reveal the specific identity of bacterial isolates (Drancourt *et al.*, 2000). Sequence analysis of the 16S rRNA gene region of *Flavobacterium* spp. can readily be used in phylogenetic comparisons, fish disease diagnostics and epidemiological studies (Bernardet *et al.*, 2002; and Darwish and Ismaiel, 2005). Amplification of the *Flavobacterium* spp. 16S rRNA gene region has been performed with both universal (Arias *et al.*, 2004; Triyanto *et al.*, 1999; and Urdaci *et al.*, 1998) and 16S species-specific primers designed specifically for the *Flavobacterium* spp. of interest (Baliarda *et al.*, 2002; Darwish *et al.*, 2004; Madetoja and Wiklund, 2002; Michel *et al.*, 2002; Triyanto and Wakabayashi, 1999; Urdaci *et al.*, 1998; and Wiklund *et al.*, 2000). A variety of *Flavobacterium* spp. isolates have been identified by amplification of 16S rRNA genes from diverse sites, including river epilithon (O'Sullivan *et al.*, 2002), paper mill slimes (Oppong *et al.*, 2003), and from fish farm water and fish tissue samples (Baliarda *et al.*, 2002; Madetoja and Wiklund, 2002; and Wiklund *et al.*, 2000). Therefore,

this technique has been described as a rapid and effective method for detection and identification of *Flavobacterium* spp. isolated from water samples (Bernardet and Bowman, 2005; Darwish *et al.*, 2004; Madetoja and Wiklund, 2002; Toyama *et al.*, 1994; Urdaci *et al.*, 1998; and Wiklund *et al.*, 2000). Bernardet *et al.* (2002), however, have proposed that 16S rRNA gene sequence analysis should be used in combination with other genomic methods for delineating newly isolated species belonging to the genus *Flavobacterium*.

Both phenotypic and genotypic typing techniques have been used to characterize *Flavobacterium* spp., especially fish pathogenic species (Bernardet and Bowman, 2005, Bernardet *et al.*, 1996; Chakroun *et al.*, 1997; Crump *et al.*, 2001, Figueiredo *et al.*, 2005; Lorenzen *et al.*, 1997; Triyanto and Wakabayashi, 1999; and Madsen and Dalsgaard, 2000). Chemotaxonomic typing techniques which have been performed on *Flavobacterium* spp. include serological typing (Crump *et al.*, 2001; and Michel *et al.*, 2002), fatty acid typing (Bernardet *et al.*, 1996; Bernardet *et al.*, 2002; and Figueiredo *et al.*, 2005), WCP typing (Bernardet *et al.*, 1996; and Bernardet *et al.*, 2002), bacteriocin typing (Bernardet and Bowman, 2005) and multilocus enzyme electrophoresis (Bernardet and Bowman, 2005). Comparison of DNA hybridization data and WCP profiles of members of the family *Flavobacteriaceae* have revealed a close correlation between high DNA homology and high similarity of WCP fingerprints (Bernardet and Bowman, 2005; and Bernardet *et al.*, 2002). Different strains of certain *Flavobacterium* species, including *F. columnare* and *F. psychrophilum*, have also shown similar WCP profiles (Bernardet *et al.*, 1996). Very little is known about the function of OMPs present in *Flavobacterium* spp. and, at present, have not been linked to virulence of pathogenic *Flavobacterium* spp. or biofilm formation.

The use of molecular-based techniques is not only restricted to the identification of specific *Flavobacterium* spp. within this genus, but also allows for the detection of intra- and interspecies variation. Molecular fingerprinting techniques may also be used for epidemiological tracing of infections caused by pathogenic *Flavobacterium* spp., such as *F. columnare* and *F. psychrophilum* (Bernardet *et al.*, 2005). Hence, this study was focussed on the identification and

discrimination of presumptive *Flavobacterium* spp. isolates based on 16S rRNA gene sequence analysis and several molecular and chemotaxonomic typing techniques. Linkage between biochemical and physiological characteristics (Chapter 2) and clusters obtained by molecular typing was also addressed.

3.2 MATERIALS AND METHODS

3.2.1 Genomic DNA Isolation

Genomic DNA of presumptive *Flavobacterium* spp. isolates was isolated by the CTAB/NaCl mini-prep protocol (Ausubel *et al.*, 1989). Overnight EAOB cultures were harvested by centrifugation and cell pellets were suspended in 567 µl TE buffer [10 mM Tris.Cl, and 1 mM EDTA, (pH 8)]. Thirty µl of SDS and 3 µl of 20 mg/ml proteinase K were added and incubated at 37°C for 1 h. After the addition of 100 µl of 5 M NaCl and 80 µl CTAB/NaCl [10% CTAB, and 0.7 M NaCl], the mixtures were incubated at 65°C for 10 min. Genomic DNA in the mixtures was extracted by phenol/chloroform/isoamyl alcohol extraction, precipitated using isopropanol and pellets were washed with 70% ethanol. The resulting pellets were resuspended in TE buffer (Ausubel *et al.*, 1989) and stored at -20°C.

3.2.2 16S rRNA Identification Of *Flavobacterium* spp. Isolates

In order to confirm the identity of isolates presumptively identified as belonging to the genus *Flavobacterium* (Chapter 2), the 16S rRNA genes of nine isolates (YO10, YO11, YO15, YO19, YO34, YO46, YO51, YO60 and YO64), were amplified using the following 16S rRNA universal primer sets: 16S-F 5'-AGTTTGATCCTGGCTCAG-3' and 16S-R 5'-TACCTTGTTACGACTTCACCCCA-3' (Heyndrickx *et al.*, 1996); F1 5'-AGAGTTTGATCITGGCTCAG-3' and R5 5'-

ACGGITACCTTGTTACGACTT-3'; and F3 5'-GCCAGCAGCCGCGGTAATAC-3' and R5 5'-ACGGITACCTTGTTACGACTT-3' (personal communication, Prof. D.E. Rawlings, University of Stellenbosch, RSA). Twenty-five μ l reaction volumes comprised of 1.5 μ M of each primer, 100 μ M dNTP's, 25 mM $MgCl_2$ (Southern Cross Biotechnology, RSA), amplification buffer (10 \times) (Southern Cross Biotechnology), 1U SuperTherm DNA polymerase (Southern Cross Biotechnology) and 1 μ l genomic DNA. Amplification was performed in a PCR Sprint thermal cycler (Hybaid, UK) under the following amplification conditions: 94°C for 3 min, followed by 35 amplification cycles of DNA denaturation at 94°C for 30s, primer annealing at 55°C for 1 min, and extension at 72°C for 1 min, and a final extension step of 72°C for 8 min.

PCR products were subjected to electrophoresis in 1% (m/v) agarose gels, in 1 \times TAE-buffer (40 mM Tris base, 20 mM glacial acetic acid, and 2 mM EDTA), stained with ethidium bromide and viewed by UV transillumination. DNA molecular weight marker VI (2176/1766/1230/1033/653/517/453/394/298/234-220/154 bp) (Roche, Germany) was used as a size marker. Fragment sizes were determined using UVIDOC V.97 (UVIttec, UK). PCR fragments with molecular weights of 1500 bp (primers 16S-F and 16S-R, and F1 and R5) and 1000 bp (primers F3 and R5), respectively, from a given genomic DNA template were considered a positive result.

The 16S rRNA amplicons of isolates YO10, YO11, YO15, YO19, YO34, YO46, YO51, YO60 and YO64 were sequenced using the ABI373A automatic sequencer (Perkin Elmer Applied Biosystem, USA). DNA sequence similarity searches were performed using the BLAST 2.9 algorithm (Altschul *et al.*, 1997). Alignments of DNA sequences to reference sequences, obtained from GenBank, and phylogenetic tree analysis were performed using DNAMAN (version 4.0) (Lynnon BioSoft, Canada).

To identify the species grouping of the study *Flavobacterium* spp. isolates, the 16S rRNA genes of all 32 study isolates and bacteria listed in Table 3.1 were amplified using 16S rRNA gene species-specific primers: FP1 5'-GTTAGTTGGCATCAACAC-3' and FP2 5'-TCGATCCTACTTGCGTAG-3' (Urdaci

et al., 1998), and Fvp F1 5'-GCCCAGAGAAATTTGGAT-3' and Fvp R1 5'-TGCGATTACTAGCGAATCC-3' (Bader *et al.*, 2003). Twenty-five μ l reaction volumes comprised of 1.5 μ M of each primer, 100 μ M dNTP's, 25 mM MgCl₂ (Southern Cross Biotechnology), amplification buffer (10 \times) (Southern Cross Biotechnology), 1U SuperTherm DNA polymerase (Southern Cross Biotechnology) and 0.6 μ l genomic DNA. Amplification was performed in a PCR Sprint thermal cycler (Hybaid, UK) under the following amplification conditions: 94°C for 3 min, followed by 35 amplification cycles of DNA denaturation at 94°C for 30s, primer annealing at 59°C for 1 min, and extension at 72°C for 1 min, and a final extension step of 72°C for 8 min.

PCR products were subjected to electrophoresis in 1% (m/v) agarose gels, in 1 \times TAE-buffer, stained with ethidium bromide and viewed by UV transillumination. The O'GeneRuler 100 bp DNA ladder (3000/2000/1500/1200/1031/900/800/700/600/500/400/300/200/100 bp) (Fermentas, Canada) was used as a size marker. Fragment sizes were determined using UVIDOC V.97 (UVIttec, UK). The presence of a 1088 bp amplicon using primers FP1 and FP2 was indicative of *F. psychrophilum*, while that of a 1193 bp amplicon (Fvp F1 and Fvp R1 primers) was representative of *F. columnare* (Bader *et al.*, 2003) and/or *F. johnsoniae* (Darwish *et al.*, 2004).

TABLE 3.1. Bacterial isolates used in 16S rRNA species-specific amplification.

Isolates	Type strain
<i>Aeromonas</i> spp.	-*
<i>A. hydrophila</i>	-
<i>A. salmonicida</i>	-
<i>A. sobria</i>	-
<i>C. balustinum</i>	NCTC 11212
<i>C. gleum</i>	ATCC 35910
<i>C. indologenes</i>	NCTC 10796
<i>C. indoltheticum</i>	ATCC 27950
<i>C. joostei</i>	LMG 18212
<i>C. meningosepticum</i>	ATCC 13253
<i>E. coli</i>	DH5 α
<i>M. odoratus</i>	ATCC 4651
<i>S. multivorum</i>	NCTC 11343

* Laboratory isolates/strains

3.2.3 16S rRNA gene PCR-RFLP Analysis

The 1193 bp amplicons (primers Fvp F1 and Fvp R1) obtained by 16S rRNA gene species-specific amplification of all 32 study isolates (section 3.2.2), were subjected to restriction analysis using each of the following restriction endonucleases *AluI*, *CfoI*, *HaeIII*, *HinI*, *MspI*, and *TaqI*, respectively. Restriction fragments were subjected to electrophoresis in 1.5 % (m/v) agarose gels, in 1 × TAE-buffer, and viewed as described in Section 3.2.2. DNA molecular weight marker VI (2176/1766/1230/1033/653/517/453/394/298/234-220/154 bp) (Roche, Germany) or the O'GeneRuler 100-bp DNA ladder (3000/2000/1500 /1200/1031/900/800/700/600/500/400/300/200/100 bp) (Fermentas, Canada), respectively, were used as size markers. Restriction patterns were examined visually for differences in number of fragments, while differences in molecular weight of fragments were estimated using UVIDOC V.97 (UVIttec, UK).

Triyanto and Wakabayashi (1999) have assigned *F. columnare* isolates to genomovars by PCR-RFLP analysis of 1.5 kb 16S rRNA gene fragments generated by amplification using universal primers. In an effort to assign study

isolates to these previously described genomovars, the 1.5 kb PCR amplicons of five isolates (YO10, YO19, YO51, YO60 and YO64) obtained by amplification with universal primers 16S-F and 16S-R (section 3.2.2) were subjected to *Hae*III restriction (Triyanto and Wakabayashi, 1999). Restriction fragments were subjected to electrophoresis in 8% acrylamide gels [10.64 ml of 30% acrylamide (29 g acrylamide, 1 g N,N'-methylenebisacrylamide, and ddH₂O to 100 ml), 21.08 ml ddH₂O, 8 ml 5 × TBE (45 mM Tris base, 45 mM boric acid, and 1 mM EDTA), 20% ammonium persulfate, and 14 µl N,N,N',N'-tetramethylethylenediamine (TEMED)], in 1 × TBE-buffer (5 × TBE: 45 mM Tris base, 45 mM boric acid, and 1 mM EDTA), and viewed as described previously in Section 3.2.2. The O'GeneRuler 100 bp DNA ladder (3000/2000/1500/1200/1031/900/800/700/600/500/400/300/200/100 bp) (Fermentas, Canada) was used as a size marker. Molecular weight of fragments was estimated using UVIDOC V.97 (UVIttec, UK). Patterns obtained were compared to restriction profiles described by Triyanto and Wakabayashi (1999).

3.2.4 RAPD-PCR Fingerprinting

RAPD fingerprints were generated for all 32 study isolates using the 10-mer primer UBC 214 5'-CATGTGCTTG-3' (University of British Columbia, Canada). A 25 µl reaction mixture consisted of 2.5 µl reaction buffer (10×) (Southern Cross Biotechnology), 100 µM concentration of each dNTP, 0.6 µl of primer, 25 mM MgCl₂ (Southern Cross Biotechnology), 1 µl of template DNA, and 1 U SuperTherm DNA polymerase (Southern Cross Biotechnology). Amplification was performed in a PCR Sprint thermal cycler (Hybaid, UK) using the following amplification conditions: 94°C for 3 min, followed by 45 amplification cycles of DNA denaturation at 94°C for 30s, primer annealing at 34°C for 1 min, and extension at 72°C for 2 min, and a final extension step of 72°C for 10 min. Negative controls, containing no template DNA, were included in all RAPD-PCR experiments. In order to ensure reproducibility, RAPD amplifications were

consistently performed using the same thermal cycler. Additionally, isolates YO10, YO12 and YO19 were repeatedly amplified on separate occasions to confirm reproducibility of RAPD profiles. To further confirm RAPD reproducibility, genomic DNA of isolates YO12 and YO19 was isolated on 2 separate occasions and subjected to amplification. The RAPD products were subjected to electrophoresis in 2% (m/v) agarose gels, in 1 × TAE-buffer, and viewed as described previously in Section 3.2.2. DNA molecular weight marker VI (2176/1766/1230/1033/653/517/453/394/298/234-220/154 bp) (Roche, Germany) or the O'GeneRuler 100 bp DNA ladder (3000/2000/1500/1200/1031/900/800/700/600/500/400/300/200/100 bp) (Fermentas, Canada) were used as size markers. The fingerprints were visually examined for differences in number of fragments amplified, differences in molecular weight and intensity of PCR fragments. Band sizes of RAPD amplicons were calculated using UVIDOC V.97 (UVIttec, UK). RAPD profiles were analysed using GelCompar (Applied Maths BVBA, Belgium) and natural groupings of similar patterns were clustered using the Pearson product-moment correlation coefficient with global optimization and results were displayed as a dendrogram.

3.2.4 ERIC-PCR Fingerprinting

ERIC-PCR was performed using the following primers: ERIC1 5'-ATGTAAGCTCCTGGGGATTCAC-3' and ERIC2 5'-AAGTAAGTGACTGGG GTGAGCG-3' (Versalovic *et al.*, 1991). ERIC-PCR was performed as described previously (Versalovic *et al.*, 1991). A 25 µl reaction mixture consisted of 2.5 µl reaction buffer (10×) (Southern Cross Biotechnology), 100 µM concentration of each dNTP, 0.2 µl of each primer, 25 mM MgCl₂ (Southern Cross Biotechnology), 1 µl of template DNA, and 1 U SuperTherm DNA polymerase (Southern Cross Biotechnology). Amplification was performed in a PCR Sprint thermal cycler (Hybaid, UK) using the following amplification conditions: 94°C for 7 min, followed by 30 amplification cycles of DNA denaturation at 94°C for 30s,

primer annealing at 52°C for 1 min, and extension at 65°C for 8 min, and a final extension step of 65°C for 16 min. Negative controls, containing no template DNA, were included in all ERIC-PCR experiments. The PCR products were electrophoresed in 2% (m/v) agarose gel in 1 × TAE-buffer, and viewed as described previously in Section 3.2.2. DNA molecular weight marker VI (2176/1766/1230/1033/653/517/453/394/298/234-220/154 bp) (Roche, Germany) was used as a size marker. The fingerprints were visually examined for differences in number of fragments amplified, differences in molecular weight and intensity of PCR products. Band sizes of ERIC amplicons were calculated using UVIDOC V.97 (UVIttec, UK).

3.2.5 REP-PCR Fingerprinting

REP-PCR was performed using the following primers: REP1 5'-IIICGICGICATCIGGC-3' and REP2 5'-ICGICTTATCIGGCCTAC-3' (Versalovic *et al.*, 1991). Twenty-five µl reaction volumes consisted of 2.5 µl reaction buffer (10×) (Southern Cross Biotechnology), 100 µM concentration of each dNTP, 0.2 µl of each primer, 25 mM MgCl₂ (Southern Cross Biotechnology), 1 µl of template DNA, and 1 U SuperTherm DNA polymerase (Southern Cross Biotechnology). Amplification was performed in a PCR Sprint thermal cycler (Hybaid, UK) under the following amplification conditions: 94°C for 3 min, followed by 35 amplification cycles of DNA denaturation at 94°C for 30s, primer annealing at 40°C for 1 min, and extension at 65°C for 8 min, and a final extension step of 65°C for 16 min. In order to ensure reproducibility, REP amplifications were consistently performed using the same thermal cycler. Additionally, isolates YO10, YO12 and YO19 were repeatedly amplified on separate occasions to confirm reproducibility of REP profiles. To further confirm REP reproducibility, genomic DNA of isolates YO12 and YO19 was isolated on 2 separate occasions and subjected to amplification. The REP products were subjected to electrophoresis in 1.5% (m/v) agarose gels, in 1 × TAE-buffer, and viewed as described previously in Section

3.2.2. The O'GeneRuler 100 bp ladder (3000/2000/1500/1200/1031/900/800/700/600/500/400/300/200/100 bp) (Fermentas, Canada) and the O'RangeRuler 200 bp DNA ladder (3000/2800/2600/2400/2200/1800/1600/1400/1200/1000/800/600/400/200 bp) (Fermentas, Canada) were used as size markers. The fingerprints were visually examined for differences in number of fragments amplified, differences in molecular weight and intensity of PCR products. Band sizes of REP amplicons were calculated using UVIDOC V.97 (UVIttec, UK). REP profiles were analysed using GelCompar (Applied Maths BVBA, Belgium) and natural groupings of similar patterns were clustered using the Pearson product-moment correlation coefficient with global optimization and results were displayed as a dendrogram.

3.2.6 Plasmid Profiling

Plasmid DNA was isolated according to a modified Birnboim and Doly method (1979). One-and-a half ml of overnight EAOB culture was centrifuged at 12000 rpm for 1 min. Cell pellets were suspended in 100 µl Solution I [25 mM Tris.HCL, 10 mM EDTA, (pH 8), 50mM glucose, and 5 mg/ml lysozyme], vortexed and incubated at room temperature for 10 min. Two hundred µl of Solution II (0.2 N NaOH, and 1% SDS) was added, vortexed and incubated on ice for 5 min. Hundred and fifty µl of Solution III [3 M sodium acetate, and 20% v/v glacial acetic acid, (pH 4.8)] was added, gently inverted twice and incubated on ice for 20 min. The suspension was centrifuged at 12000 rpm for 20 min and 400 µl of supernatant was placed in a new tube. Nine hundred µl of cold ethanol was added, inverted and incubated at -20°C for 60 min. The suspension was centrifuged at 12000 rpm for 10 min. The pellet was suspended in 100 µl of Solution IV (3 M sodium acetate, and 1 M Tris) and 200 µl cold ethanol, inverted twice and incubated at -20°C for 10 min. The suspension was centrifuged at 12000 rpm for 10 min, the pellet was air dried and resuspended in 20 µl TE buffer [10 mM Tris.HCL, and 1 mM EDTA, (pH 8)]. Plasmids were separated in 1%

(m/v) agarose gels in 1 × TAE-buffer, and viewed as described previously in Section 3.2.2. A plasmid from *Escherichia coli* strain JM105 containing pUCBM21 (~3 kb) was used as a size marker. Plasmid profiles were identified following visual examination for differences in size and band intensity. The molecular weight of plasmids was calculated using UVIDOC V.97 (UVItec, UK).

3.2.7 SDS-PAGE of WCP Samples

Whole-cell proteins were prepared with *N*-lauryl-sarcosine using a modification of the method described by Sawai *et al.* (1982). Fifty ml of overnight EAOB cultures were centrifuged at 12000 rpm for 15 min. Cells were washed with 10 ml PBS (pH 7), centrifuged at 12000 rpm for 15 min, and resuspended in 10 ml of PBS buffer. Cells were sonicated using a Sonicator™ Cell Disruptor (Heat Systems-Ultrasonics Inc, USA) and 2 ml aliquots were stored at -20°C as whole cell protein preparations.

Protein preparations, in 50 µl volumes, were solubilised in 20 µl 2 × sample buffer [25 ml 4 × Tris-Cl/SDS (pH 6.8), 20 ml glycerol, 4 g SDS, 2 ml 2-mercaptoethanol, and 1 mg bromophenol blue, ddH₂O to 100 ml), heated for 5 min at 100°C and electrophoresed in a 12% polyacrylamide gel by SDS-PAGE at 5 A for 14 h. A PageRuler™ prestained protein ladder (170/130/100/70/55/45/35/25/15/10 kDa) (Fermentas, Canada) was used as protein marker. Proteins were stained with silver staining, using a modified procedure described by Tsai and Frasch (1982).

SDS-PAGE gels were immersed in fixing solution (50% methanol, 12% acetic acid, and 0.0185% formaldehyde) for 1 h. This was followed by 2 × 10 min washes in wash solution (50% ethanol). Thereafter, gels were pretreated in 2% sodium thiosulphate solution for 5 min and washed (3 × 30 s) in sterile ddH₂O. Gels were stained for 10 min in a silver nitrate solution (0.2% silver nitrate and 0.028% formaldehyde) and then washed (2 × 20 s) in sterile double-distilled water. This procedure was performed at room temperature with gentle agitation.

Developer (6% sodium carbonate, 0.0185% formaldehyde, and 0.14 mg sodium thiosulphate) was added thereafter, and gels were agitated gently until protein bands appeared. The developer was poured off and gels stored in stop solution (50% methanol and 12% acetic acid) after sufficient development of protein bands.

Gels were photographed with a Canon PowerShot A95 digital camera (Canon Inc., Japan) and fixed in cellophane using a Gel Air Dryer (Bio-Rad laboratories, USA). The protein profiles were visually examined for differences in number, molecular weight and intensity of protein bands. Protein band sizes were calculated using UVIDOC V.97 (UVItec, UK). WCP profiles were analysed using GelCompar (Applied Maths BVBA, Belgium) and natural groupings of similar patterns were clustered using the Pearson product-moment correlation coefficient with global optimization and results were displayed as a dendrogram.

3.2.8 SDS-PAGE of OMP Samples

Outer membrane proteins were prepared with *N*-lauryl-sarcosine using a modification of the method described by Benedí and Martínez- Martínez (2001). Fifty ml of overnight EAOB cultures were centrifuged at 12000 rpm for 15 min. Cells were washed with 10 ml PBS (pH 7), centrifuged at 12000 rpm for 15 min, and resuspended in 10 ml of PBS buffer. Cells were sonicated using a Sonicator[™] Cell Disruptor (Heat Systems-Ultrasonics Inc, USA) and centrifuged at 6000 rpm for 10 min. The supernatant was centrifuged at 37000 rpm for 1 h at 4°C in an L7-65 Ultracentrifuge (Beckman Instruments Inc, USA). The pellet was suspended in 2 ml of 2% sodium lauryl sarcosine (SLS) in PBS and incubated at room temperature for 30 min. Suspensions were centrifuged at 37 000 rpm for 45 min at 4°C. The pellet was washed with 1% SLS in PBS and centrifuged at 37 000 rpm for 40 min at 4°C and subsequently used as partially purified OMP preparations. OMPs were resuspended in sterile ddH₂O and stored at -20°C.

Protein preparations were solubilised in 20 μ l 2 \times sample buffer [25 ml 4 \times Tris-Cl/SDS (pH 6.8), 20 ml glycerol, 4 g SDS, 2 ml 2-mercaptoethanol, and 1 mg bromophenol blue, ddH₂O to 100 ml), heated for 5 min at 100°C and electrophoresed in 12% polyacrylamide gels by SDS-PAGE at 5 A for 14 h. A PageRuler™ prestained protein ladder (170/130/100/70/55/45/35/25/15/10 kDa) (Fermentas, Canada) was used as protein marker. Proteins were stained with silver staining, using the modified procedure described by Tsai and Frasch (1982) as described previously in Section 3.2.8.

Gels were photographed with a Canon PowerShot A95 digital camera (Canon Inc., Japan) and fixed in cellophane using Gel Air Dryer (Bio-Rad laboratories, USA). The protein profiles were visually examined for differences in number, molecular weight and intensity of protein bands. Protein band sizes were calculated using UVIDOC V.97 (UVIttec, UK). OMP profiles were analysed using GelCompar (Applied Maths BVBA, Belgium) and natural groupings of similar patterns were clustered using the Pearson product-moment correlation coefficient with global optimization and results were displayed as a dendrogram.

3.2.9 Typeability, Reproducibility and Discrimination

Data obtained by the typing techniques were evaluated according to the following criteria: typeability, reproducibility and discrimination (Chapter 1, Section 1.6). The Simpsons index of diversity was used to calculate the discriminatory index of a given typing technique (Simpson, 1949 – cited by Hunter and Gaston, 1988).

3.3 RESULTS

3.3.1 16S rRNA Identification Of *Flavobacterium* spp. Isolates

The universal primer sets 16S-F + 16S-R, and F1 + R5 yielded the expected 1500 bp amplification products for all the presumptive *Flavobacterium* spp. isolates tested (Fig 3.1). The universal primers F3 and R5 also yielded the expected 1000 bp amplification products for all the isolates tested (data not shown).

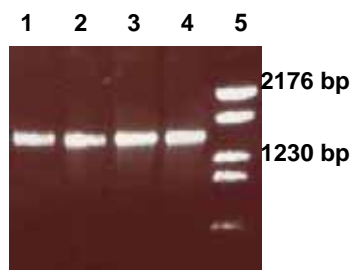


FIG. 3.1. Agarose gel electrophoresis of 16S rRNA PCR products of *Flavobacterium* spp. isolates DNA amplified with primer sets 16S-F + 16S-R, and F1 + R5. Lanes 1 and 2: isolates YO10 and YO19 amplified with primers 16S-F and 16S-R, respectively; lanes 3 and 4: isolates YO10 and YO19 amplified with primers F1 + R5, respectively, and lane 5: molecular weight marker VI [Roche, Germany (2176/1766/1230/1033/653/517/453/394/298/234-220/154 bp)].

Alignments of the 16S rRNA gene sequences (Appendix 1) of nine selected *Flavobacterium* spp. study isolates (Fig. 3.2) revealed the following:

- a) Sequences of isolates YO10, YO11 and YO15 shared 100% homology and 99% homology to that of isolate YO46.
- b) Sequences of isolates YO19, YO34 and YO64 shared 99% homology and 98% homology to that of isolates YO51 and YO60, respectively.
- c) The 2 clusters showed 97% sequence homology.

On comparison with sequences selected from the GenBank database, the 1st cluster of isolates YO10, YO11, YO15 and YO46 showed strongest sequence homology to sequences from *F. johnsoniae* (AB078043) (Welker *et al.*, 2005), *F. hydatidis* (AY753068) (Welker *et al.*, 2005) and a sequence from a *Flavobacterium* spp. isolated from a river, *Flavobacterium* spp. WUBA46 strain (AF336355) (Bohnert *et al.*, 2001) (Figs. 3.2-3.3). The 2nd cluster of isolates YO19, YO34, YO64, YO51 and YO60 shared 98% sequence homology to a 16S rRNA gene sequence obtained from a *Flavobacterium* spp. isolated from an intestinal sample of rainbow trout (AY374109) (Huber *et al.*, 2004) (Fig. 3.2). Both clusters of study isolates shared 95% sequence homology to sequences from *F. aquatile*, *F. branchiophilum* and *F. psychrophilum* (Fig. 3.3) and 92% homology to *F. columnare* sequences, respectively (Appendix 2, Fig. A).

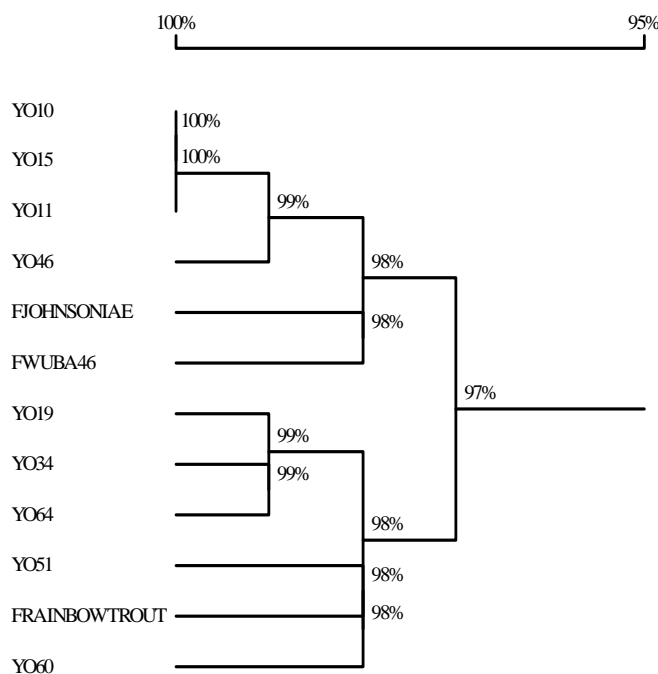


FIG. 3.2. 16S rRNA gene sequence homology tree showing 2 clusters of nine selected *Flavobacterium* spp. study isolates YO10, YO11, YO15 + YO46 and YO19, YO34, YO64, YO51 + YO60, and FJOHNSONIAE AB078043 (Welker *et al.*, 2005), FRAINBOWTROUT AY374109 (Huber *et al.*, 2004), and FWUBA46 AF336355 (Bohnert *et al.*, 2001).

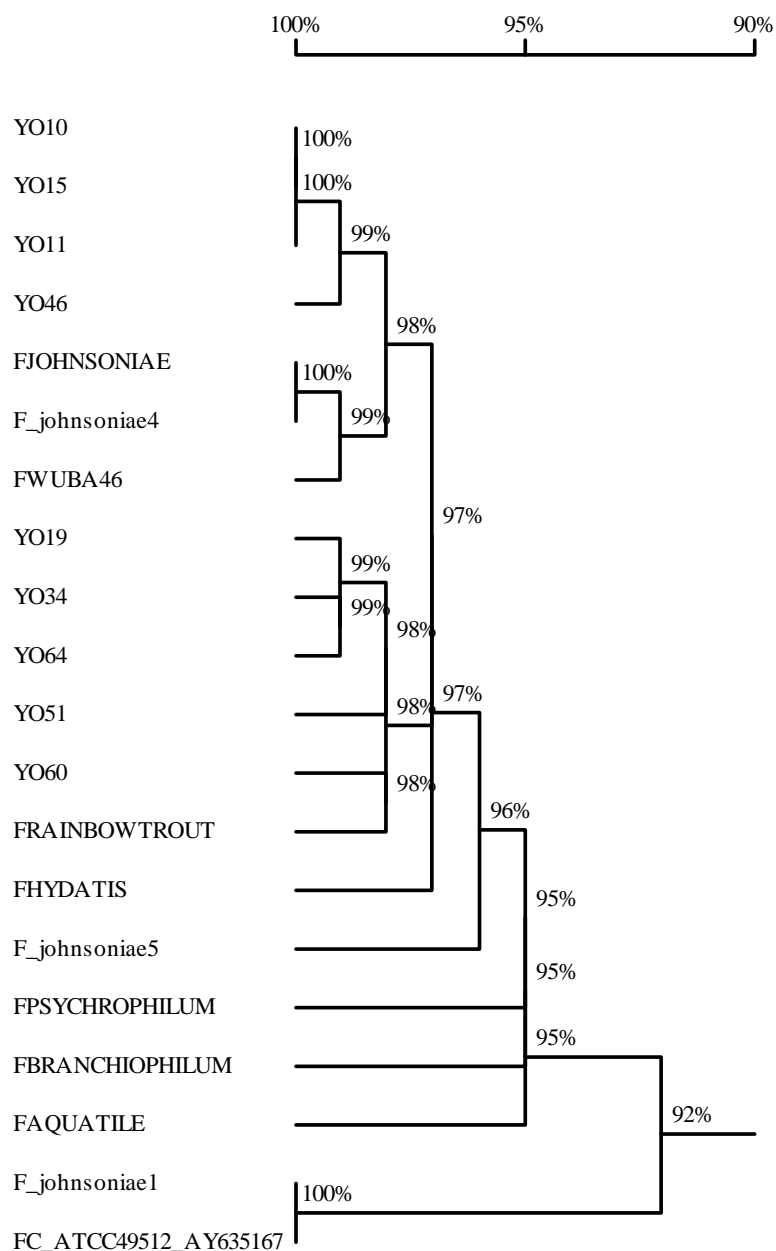


FIG. 3.3. 16S rRNA sequence homology tree showing relatedness of nine selected *Flavobacterium* spp. study isolates to other related bacteria in the genus *Flavobacterium*: FAQUATILE M62797 (Woese *et al.*, 1990), FBRANCHIOPHILUM D14017 (Nakagawa and Yamasato, 1996), FC ATCC49512 AY635167 (Darwish and Ismaiel, 2005), FHYDATIS AY753068 (Welker *et al.*, 2005), Fjohnsoniae1 AY635167 (Darwish and Ismaiel, 2005), Fjohnsoniae4 M59053 (Ghera and Woese, 1992), Fjohnsoniae5 AY753067 (Welker *et al.*, 2005), FJOHNSONIAE AB078043 (Nakagawa *et al.*, 2002), FPSYCHROPHILUM AB07860 (Nakagawa *et al.*, 2002), FRAINBOWTROUT AY374109 (Huber *et al.*, 2004), and FWUBA46 AF336355 (Bohnert *et al.*, 2001), based on comparison of 16S rRNA gene sequences.

None of the 32 *Flavobacterium* spp. isolates produced the 1088 bp amplicon using the *F. psychrophilum* species-specific primers FP1 and FP2 (Fig. 3.4). Of the additional bacterial strains listed in Table 3.1, a 1088 bp amplicon, was obtained with *C. balustinum* NCTC 11212 (Fig. 3.4; lane 11). Non-specific amplification was observed for the following bacterial strains: *C. balustinum* NCTC 11212, *C. gleum* NCTC 11432, *C. joostei* LMG 18212, and *M. odoratus* NCTC 11036 (Fig. 3.4).

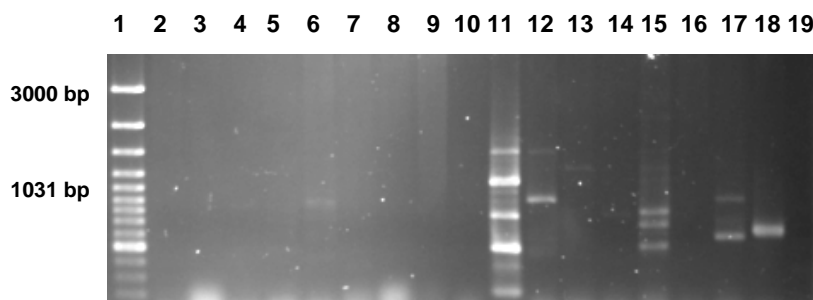


FIG. 3.4. Agarose gel electrophoresis of 16S rRNA PCR products of *Flavobacterium* spp. isolates and various type species DNA with the *F. psychrophilum* species-specific primer set FP1 + FP2. Lane 1: O'GeneRuler 100 bp DNA ladder [Fermentas, Canada (3000/2000/1500/1200/1031/900/800/700/600/500/400/ 300/200/100 bp)]; lanes 2-19: isolates YO10, YO19, YO51, YO60, YO64, *E. coli* strain DH5 α , *Aeromonas* spp. isolate, *A. hydrophila*, *A. salmonicida*, *A. sobria*, *C. balustinum* NCTC 11212, *C. gleum* NCTC 11432, *C. indologenes* NCTC 10796, *C. indoltheticum* ATCC 27950, *C. joostei* LMG 18212, *C. meningosepticum* NCTC 10016, *M. odoratus* NCTC 11036, and *S. multivorum* NCTC 11343, respectively.

All the isolates presumptively identified as *Flavobacterium* spp. according to physiological and biochemical characterisation yielded the expected 1193 bp amplification product using the *F. columnare*/*F. johnsoniae* species-specific primer set, Fvp F1 + Fvp R (Fig. 3.5).

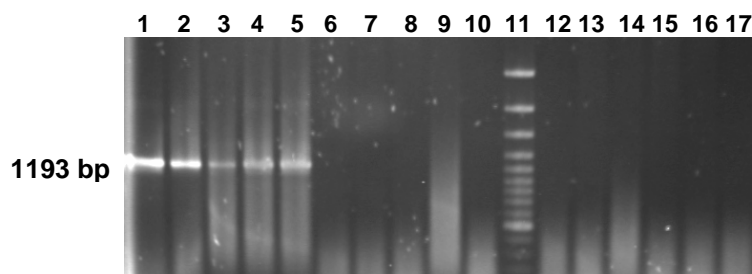


FIG. 3.5. Agarose gel electrophoresis of 16S rRNA PCR products of *Flavobacterium* spp. isolates and various type species DNA with the *F. columnare*/*F. johnsoniae* primer set FvP F1 + FvP R1. Lanes 1-10: isolates YO10, YO19, YO51, YO60, YO64, *E. coli* strain DH5 α , *Aeromonas* spp. isolate, *A. hydrophila*, *A. salmonicida*, *A. sobria*; lanes 12-17: *C. balustinum* NCTC 11212, *C. gleum* NCTC 11432, *C. indologenes* NCTC 10796, *C. indoltheticum* ATCC 27950, *C. joostei* LMG 18212, and *C. meningosepticum* NCTC 10016, respectively, and lane 11: O'GeneRuler 100 bp DNA ladder [Fermentas, Canada (3000/2000/1500/1200/1031/900/800/700/600/500/400/300/200/100 bp)].

3.3.2 16S rRNA PCR-RFLP Analysis

AluI, *HaeIII* and *HinfI* digestion of the 1193 bp 16S rRNA gene amplicon yielded a single profile, respectively, consisting of 3-4 fragments (Table 3.2, Figs. 3.6-3.8). The *AluI* restriction profile obtained for the 32 *Flavobacterium* spp. study isolates (Fig. 3.6) contained 2 additional bands (~470 bp and ~250 bp) of slightly lighter intensity compared to the bands listed in Table 3.2. These additional bands did not add up to the original 1193 bp amplicon if included with the listed bands and were probably due to partial digestion of the 1193 bp amplicon.

Additional fragments may have been lost during electrophoresis due to their small size, as not all the bands observed in the different restriction patterns added up to the original 1193 bp and 1500 bp amplicons, respectively. The rough estimation of band sizes may also have contributed to the inaccuracy of sum totals of band sizes obtained in the different restriction patterns.

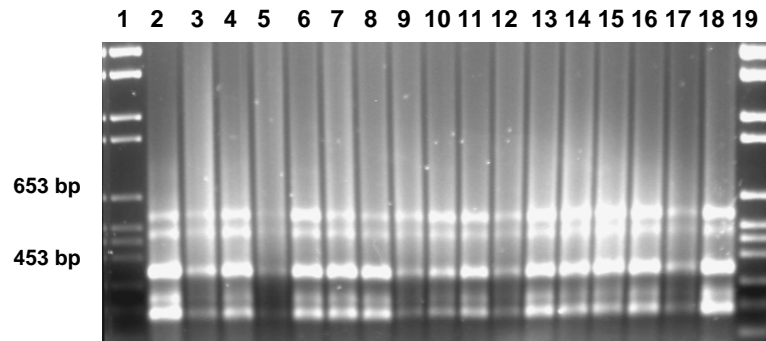


FIG. 3.6. Agarose gel electrophoresis of restriction fragments following digestion of the 1193 bp 16S rRNA amplicon with *AclI*. Lanes 1 and 19: molecular weight marker VI [Roche, Germany (2176/1766/1230/1033/653/517/453/394/298/234-220/154 bp)]; lanes 2-18: isolates YO10, YO49, YO50, YO51, YO52, YO53, YO54, YO55, YO56, YO57, YO58, YO59, YO60, YO61, YO62, YO63 and YO64, respectively.

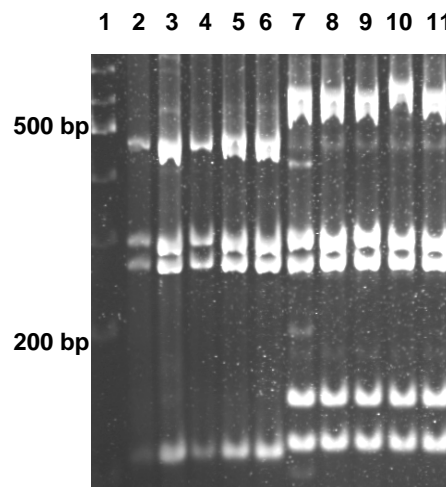


FIG. 3.7. Non-denaturing polyacrylamide gel electrophoresis of restriction fragments following digestion of the 1193 bp and 1500 bp 16S rRNA amplicons, respectively, with *HaeIII*. Lane 1: O'GeneRuler 100 bp DNA ladder [Fermentas, Canada (3000/2000/1500/1200/1031/900/800/700/600/500/400/300/200/100 bp)]; lanes 2-6: RFLP profiles of the 1193 amplicon of isolates YO10, YO19, YO51, YO60 and YO64, respectively, and lanes 7-11: RFLP profiles of the 1500 bp amplicon of isolates YO10, YO19, YO51, YO60 and YO64, respectively.

Two different patterns were observed following restriction of the 1500-bp amplicon using restriction enzyme *Hae*III (Table 3.1, Fig. 3.7). Neither of these two patterns corresponded to the *F. columnare* genomovars previously described by Triyanto and Wakabayashi (1999).

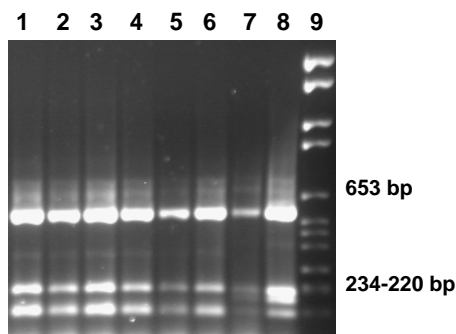


FIG. 3.8. Agarose gel electrophoresis of restriction fragments following digestion of the 1193 bp 16S rRNA amplicon with *Hinf*I. Lanes 1-8: isolates YO10, YO49, YO50, YO51, YO52, YO53, YO54, YO55, YO56, YO57, YO58, YO59, YO60, YO61, YO62, YO63 and YO64, respectively, and lane 9: molecular weight marker VI [Roche, Germany (2176/1766/1230/1033/653/517/453/394/298/234-220/154 bp)].

*Cfo*I, *Msp*I and *Taq*I restriction yielded 2 patterns each consisting of 2-3 fragments each (Table 3.2, Figs. 3.9-3.12).

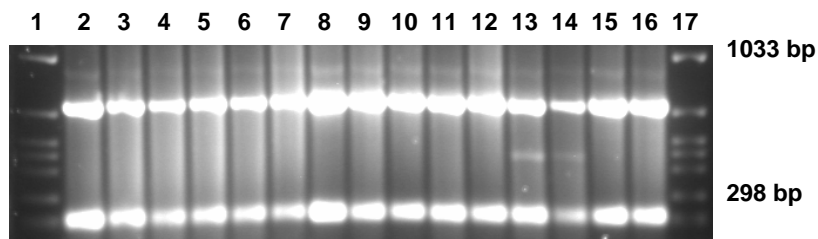


FIG. 3.9. Agarose gel electrophoresis of restriction fragments following digestion of the 1193 bp 16S rRNA amplicon with *Cfo*I. Lanes 1 and 17: molecular weight marker VI [Roche, Germany (2176/1766/1230/1033/653/517/453/394/298/234-220/154 bp)]; lanes 2-16: isolates YO10, YO11, YO12, YO15, YO19, YO20, YO21, YO26, YO34, YO35, YO38, YO45, YO46, YO49 and YO50, respectively.

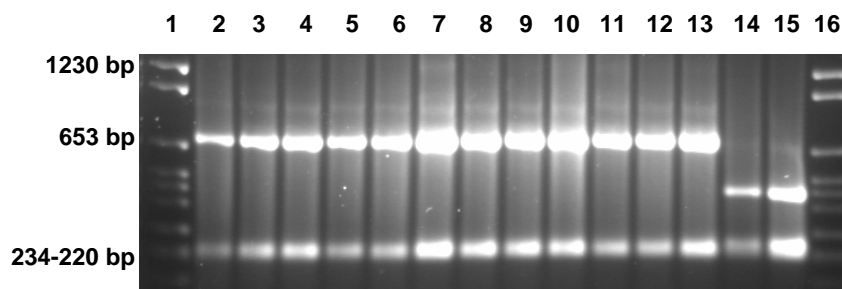


FIG. 3.10. Agarose gel electrophoresis of restriction fragments following digestion of the 1193 bp 16S rRNA amplicon with *Cfol*. Lanes 1 and 16: molecular weight marker VI [Roche, Germany (2176/1766/1230/1033/653/517/453/394/298/234-220/154 bp)]; lanes 2-16: isolates YO51, YO52, YO53, YO54, YO55, YO56, YO57, YO58, YO59, YO60, YO61, YO62, YO63 and YO64, respectively.

Following *Cfol* digestion, only isolates YO63-YO64 produced profiles of 453 + 234 bp (Fig. 3.10, Lanes 14-15). However, it was interesting to note that the *Cfol* PCR-RFLP profiles of isolates YO45 and YO46 also possessed a faint 453 bp band (Fig. 3.9, Lanes 13-14), in addition to the 690 bp and 234 bp bands.

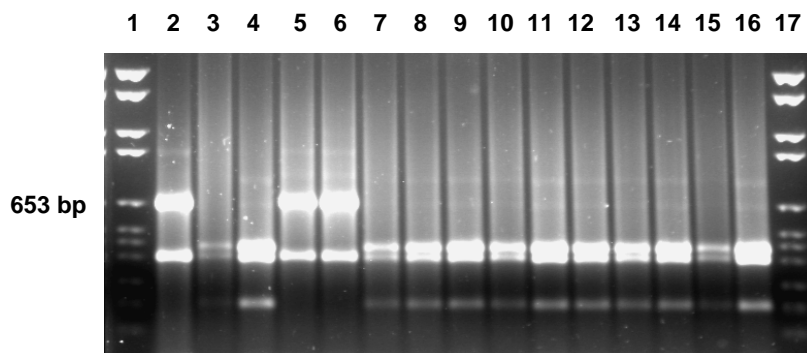


FIG. 3.11. Agarose gel electrophoresis of restriction fragments following digestion of the 1193 bp 16S rRNA amplicon with *Mspl*. Lanes 1 and 17: molecular weight marker VI [Roche, Germany (2176/1766/1230/1033/653/517/453/394/298/234-220/154 bp)]; lanes 2-16: isolates YO10, YO51, YO52, YO53, YO54, YO55, YO56, YO57, YO58, YO59, YO60, YO61, YO62, YO63 and YO64, respectively.

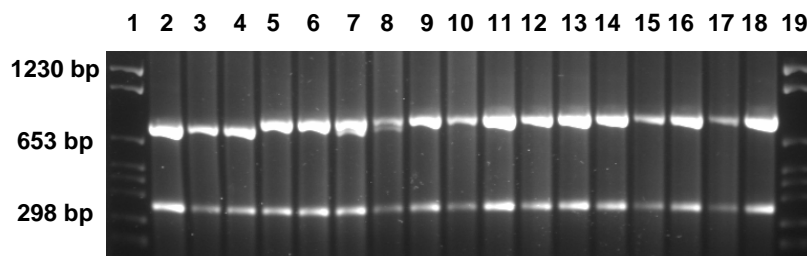


FIG. 3.12. Agarose gel electrophoresis of restriction fragments following digestion of the 1193 bp 16S rRNA amplicon with *TaqI*. Lanes 1 and 19: molecular weight marker VI [Roche, Germany (2176/1766/1230/1033/653/517/453/394/298/234-220/154 bp)]; lanes 2-18: isolates YO10, YO49, YO50, YO51, YO52, YO53, YO54, YO55, YO56, YO57, YO58, YO59, YO60, YO61, YO62, YO63 and YO64, respectively.

Prominent clusters formed by PCR-RFLP analysis included isolates YO10-YO15 + YO49-YO50 (*MspI* and *TaqI* restriction), and isolates YO19-YO38 + YO51-YO52 + YO55-YO67 (*MspI* and *TaqI* restriction), respectively (Table 3.2). PCR-RFLP profiles generated using restriction enzymes *CfoI*, *MspI* and *TaqI* permitted preliminary differentiation of the 32 *Flavobacterium* spp. study isolates to a certain degree.

TABLE 3.2. Restriction profiles of *Flavobacterium* spp. isolates following independent cleavage of the 16S rRNA amplicons using restriction enzymes *AluI*, *CfoI*, *HaeIII*, *HinfI*, *MspI*, and *TaqI*.

Restriction enzyme	16S rRNA PCR product (bp)		Number of patterns	Number of fragments	Fragment sizes (bp)	Isolates
	16S rRNA species-specific amplicon	16S rRNA universal amplicon				
<i>AluI</i>	1193		1	3	580 + 350 + 220	YO10-YO67
<i>HaeIII</i>	1193		1	4	480 + 300 + 280 + 120	YO10-YO67
		1500	2	5	620 + 300 + 280 + 155 + 128	YO10, YO19, YO51 and YO64
		1500		5	635 + 300 + 280 + 155 + 128	YO60
<i>HinfI</i>	1193		1	3	500 + 300 + 120	YO10-YO67
<i>CfoI</i>	1193		2	2	453 + 234	YO63-YO64
	1193			2	690 + 234	YO10-YO62 and YO65-YO67
<i>MspI</i>	1193		2	2	653 + 410	YO10-YO15, YO49-YO50 and YO53-YO54
	1193			3	430 + 410 + 240	YO19-YO46, YO51-YO52 and YO55-YO67
<i>TaqI</i>	1193		2	2	670 + 370	YO10-YO15 and YO45-YO50
	1193			2	750 + 370	YO19-YO38 and YO51-YO67

3.3.3 RAPD Analysis

RAPD fingerprinting with the UBC 214 primer produced 16 reproducible profiles (Figs. 3.13-3.15). Fingerprints consisted of 7 to 24 fragments ranging in size from approximately 3840 bp to 237 bp (Table 3.3). Prominent fragments, approximately 1030 bp and 770 bp in size, were shared by a large number of isolates, including isolates YO10-YO38, and isolates YO49-YO67. These identical bands, however, differed among the isolates with respect to band intensity (Figs. 3.13-3.15). An 870 bp fragment was common to RAPD fingerprints of all the isolates (Figs. 3.13-3.15). Fragments of approximately 1030 bp, 950 bp and 870 bp formed part of a prominent cluster of bands in the RAPD fingerprints of isolates YO10-YO15 (Fig 3.13, Lanes 2-5), and isolates YO49-YO62 (Fig. 3.13, Lanes 17-18; Fig. 3.14, Lanes 3-6, Lanes 8-12 and Lanes 14-18), although band intensities differed slightly.

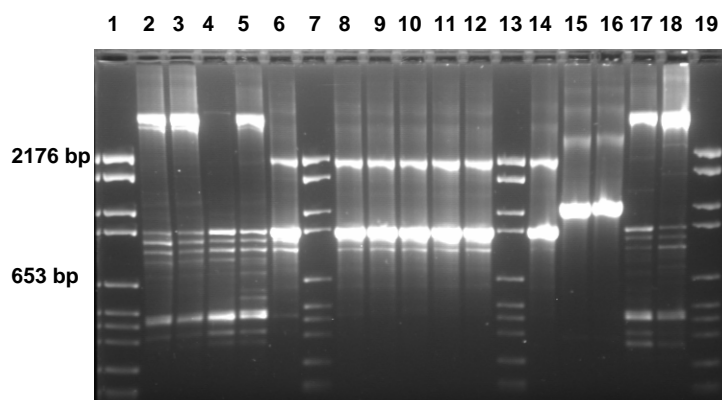


FIG. 3.13. Agarose gel electrophoresis of RAPD amplification products (UBC 214 primer) of *Flavobacterium* spp. isolates. Lanes 1, 7, 13, and 19: molecular weight marker VI [Roche, Germany (2176/1766/1230/1033/653/517/453/394/298/234-220/154 bp)]; lanes 2-6: isolates YO10, YO11, YO12, YO15 and YO19, respectively; lanes 8-12: isolates YO20, YO21, YO26, YO34 and YO35, respectively, and lanes 14-18: isolates YO38, YO45, YO46, YO49 and YO50, respectively.

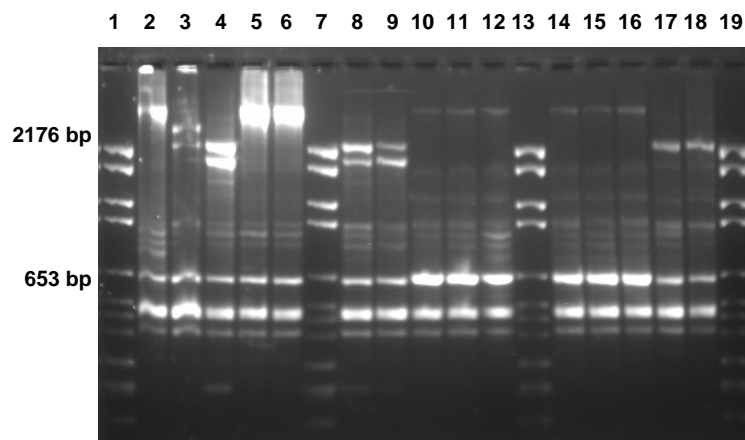


FIG. 3.14. Agarose gel electrophoresis of RAPD amplification products (UBC 214 primer) of *Flavobacterium* spp. isolates. Lanes 1, 7, 13, and 19: molecular weight marker VI [Roche, Germany (2176/1766/1230/1033/653/517/453/394/298/234-220/154 bp)]; lanes 2-6: isolates YO10, YO51, YO52, YO53 and YO54, respectively; lanes 8-12: isolates YO55, YO56, YO57, YO58 and YO59, and lanes 14-18: isolates YO60, YO61, YO62, YO63 and YO64, respectively.

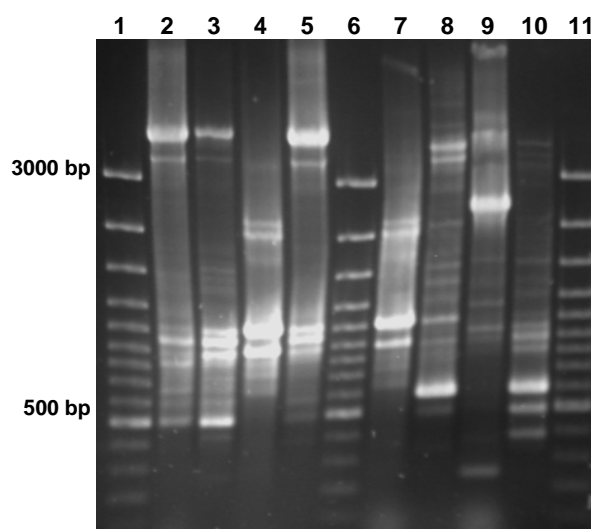


FIG. 3.15. Agarose gel electrophoresis of RAPD amplification products (UBC 214 primer) of *Flavobacterium* spp. isolates. Lanes 1, 6, and 11: O'GeneRuler 100 bp DNA ladder [Fermentas, Canada (3000/2000/1500/1200/1031/900/800/700/600/500/400/300/200/100 bp)]; lanes 2-5: isolates YO10 (repeat amplification), YO12 (repeat amplification), YO19 (repeat amplification), and YO12 (re-isolated genomic DNA), respectively, and lanes 7-10: isolates YO19 (re-isolated genomic DNA), YO65, YO66, and YO67, respectively.

TABLE 3.3. Number and sizes of RAPD-PCR products obtained from 32 *Flavobacterium* spp. isolates following amplification with the UBC 214 primer.

[illegible]

* C1-C7 represent clusters of identical/highly similar (dissimilarity < 4 bands) RAPD profiles

Of the 16 profiles, 10 major visual clusters were identified (Table 3.3). The following 11 isolates shared identical RAPD profiles in each respective cluster: cluster 1 (C1) - 4 isolates (YO10-YO11 + YO53-YO54), cluster 6 (C6) - 2 isolates (YO55-YO56), and cluster 7 (C7) - 5 isolates (YO57-YO58 + YO59-YO62), respectively (Table 3.3). In clusters 2 (C2), 3 (C3) and 9 (C9) all the isolates in each respective cluster shared the same RAPD profile (Table 3.3). The RAPD profiles obtained for 2 isolates YO51 and YO66, respectively, were unique and formed separate clusters, cluster 5 (C5) and cluster 10 (C10), respectively (Table 3.3). Repeat amplification of isolates YO10, YO12 and YO19 on separate occasions produced reproducible profiles (Fig. 3.15, Lanes 2-4). When new genomic isolations of isolates YO12 and YO19 were used as template DNA in repeat amplification, reproducible results were also obtained (Fig. 3.15, Lanes 5 + 7). DNA polymorphisms obtained in the RAPD profiles indicated a high degree of genetic diversity and allowed preliminary differentiation of *Flavobacterium* spp. study isolates.

Clusters obtained by the Pearson product-moment correlation coefficient are shown in a dendrogram (Fig. 3.16). With the UBC 214 primer, a total number of 30 clusters were established, at a maximum similarity level of 99.5%. The collection of isolates was divided into 2 major clusters (A and B) at a similarity level of 26% (Fig. 3.16). Clusters A and B were further subdivided into 16 and 13 clusters, respectively, comprising of isolates which had also been clustered visually. Although none of the isolates were clustered at 100% similarity, identical patterns were observed during visual analysis. Due to differences in band intensities and gel alignment, clustering in GelCompar appeared to group isolates differently compared to visual clusters. Isolates sharing identical RAPD profiles showed different levels of similarity, i.e., YO10-YO11 (C1) clustered at > 98% similarity, YO19-YO38 (C2) grouped at > 95% similarity, YO45-YO46 (C3) clustered at 99% similarity, YO53-YO54 (C1) clustered at > 98% similarity, YO55-YO56 (C6) clustered at 91% similarity, YO57-YO58 + YO60-YO62 (C7), grouped at 96% similarity, and YO65 + YO67 (C9) clustered at 74% similarity,

respectively (Fig. 3.16). As observed in visual analysis, the two isolates YO51 and YO66 formed single clusters.

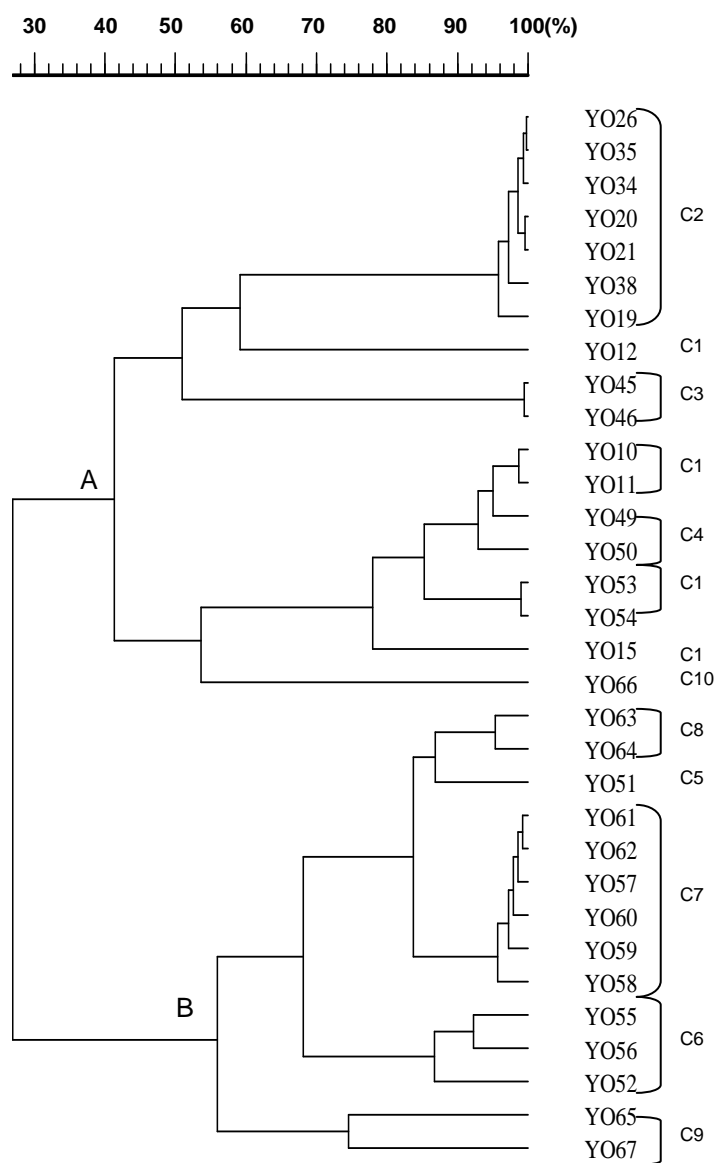


FIG. 3.16. Dendrogram of the cluster analysis of RAPD profiles of *Flavobacterium* spp. isolates following DNA amplification using random primer UBC 214.

3.3.4 ERIC PCR Analysis

Preliminary screening of isolates using the ERIC primer set ERIC1 and ERIC2 resulted in ERIC fingerprints consisting of 3 to 6 fragments ranging in size from 920 bp to 321 bp. Too few PCR amplicons were formed, and the ERIC profiles were not suitable for adequate discrimination of isolates. Thus ERIC fingerprinting was not selected for further analysis of the genetic diversity of *Flavobacterium* spp. isolates.

3.3.5 REP-PCR Analysis

REP fingerprinting with primer set REP1 and REP2 produced 18 reproducible profiles (Figs. 3.17-3.19). REP fingerprints consisted of 6 to 24 fragments ranging in size from approximately 4540 bp to 400 bp (Table 3.3). A large fragment, approximately 3310 bp in size, was shared by a large number of isolates, including isolates YO10-YO15 (Fig. 3.17, Lanes 2-5), isolates YO45-YO50 (Fig. 3.17, Lane 14, and Fig. 3.18, Lanes 2-4), and isolates YO52-YO62 (Fig. 3.18, Lanes 6-7, Lanes 9-14 and Lanes 16-18). A smaller fragment, approximately 590 bp in size, was also shared by a large number of isolates, including isolates YO12-YO38 (Fig. 3.17, Lanes 4-7 and Lanes 9-13) isolates YO49-YO56 (Fig. 3.18, Lanes 3-7 and Lanes 10-11) and isolates YO63-67 (Fig. 3.18, Lanes 19-20 and Fig. 3.19, Lanes 5-7). These prominent bands, however, differed among the isolates with respect to band intensity. No prominent band clusters were observed in REP profiles of *Flavobacterium* spp. isolates.

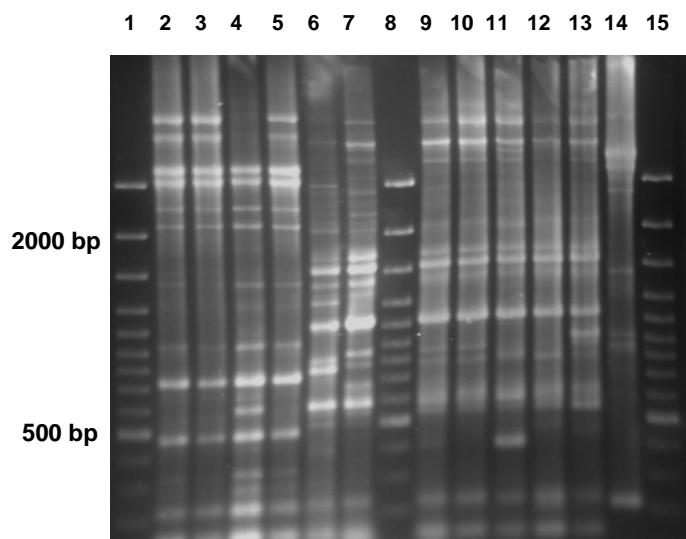


FIG. 3.17. Agarose gel electrophoresis of REP-PCR fragments of *Flavobacterium* spp. isolates. Lanes 1, 8, and 15: O'GeneRuler 100 bp DNA ladder [Fermentas, Canada (3000/2000/1500/1200/1031/900/800/700/600/500/400/300/200/100 bp)]; lanes 2-7: isolates YO10, YO11, YO12, YO15, YO19, and YO20, respectively, and lanes 9-14: isolates YO21, YO26, YO34, YO35, YO38 and YO45, respectively.

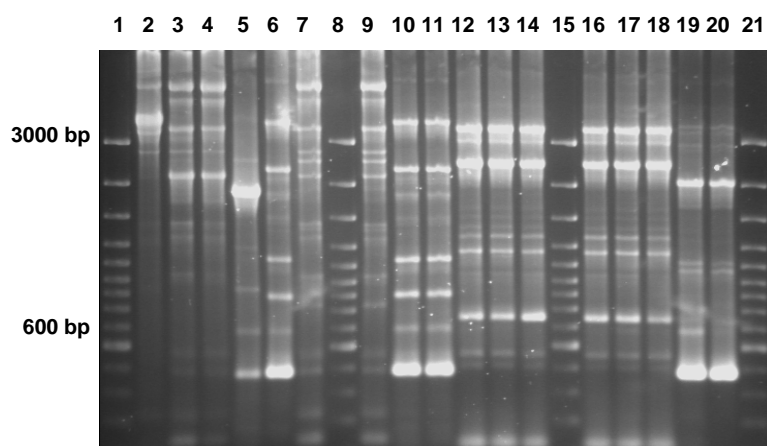


FIG. 3.18. Agarose gel electrophoresis of REP-PCR fragments of *Flavobacterium* spp. isolates. Lanes 1, 8, 15 and 21: O'GeneRuler 100 bp DNA ladder [Fermentas, Canada (3000/2000/1500/1200/1031/900/800/700/600/500/400/300/200/100 bp)]; Lanes 2-7: isolates YO46, YO49, YO50, YO51, YO52 and YO53, respectively, lanes 9-14: isolates YO54, YO55, YO56, YO57, YO58 and YO59, respectively, and lanes 16-20: isolates YO60, YO61, YO62, YO63 and YO64, respectively.

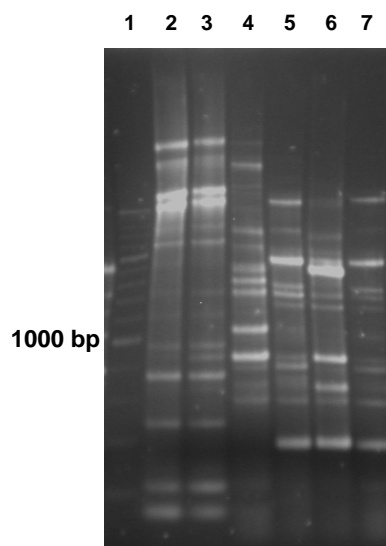


FIG. 3.19. Agarose gel electrophoresis of REP-PCR fragments of *Flavobacterium* spp. isolates. Lanes 1 and 9: O'RangeRuler 200 bp DNA ladder [Fermentas, Canada (3000/2800/2600/2400/2200/2000/1800/1600/1400/1200/1000/800/600/400/200 bp)], and lanes 2-8: YO10 (repeat amplification), YO12 (re-isolated genomic DNA), YO19 (re-isolated genomic DNA), YO65, YO66, and YO67, respectively.

Of the 18 profiles, 13 major visual clusters were identified (Table 3.4). The following six isolates shared identical REP profiles in each respective cluster: REP cluster 2 (RC2) - 4 isolates (YO12-YO15 and YO53-YO54), and REP cluster 5 (RC5) - 2 isolates (YO21-YO26), respectively (Table 3.4). In REP clusters 1 (RC1), 6 (RC6), 7 (RC7), 9 (RC9), 10 (RC10), 11 (RC11), and 12 (RC12) all the isolates in each respective cluster shared the same REP profile (Table 3.4). The REP profiles obtained for 4 isolates YO19, YO20, YO51 and YO66, respectively, were unique and formed separate clusters (Table 3.4). REP profiles of isolates clustered in RC1, RC2, and RC7 shared many common bands (Table 3.4). Similarly, REP profiles of isolates clustered in RC3, RC4 and RC5, also shared many common bands (Table 3.4). Repeat amplification of all the isolates on separate occasions produced reproducible profiles (Fig. 3.19, Lane 2). New genomic isolations of isolates YO12 and YO19 used as template DNA in repeat amplification also produced reproducible results (Fig 3.19, Lanes 3-4).

TABLE 3.4. Number and sizes of REP-PCR products obtained from 32 *Flavobacterium* spp. isolates following amplification with REP1 and REP2 primers.

[illegible]

*

RC1-RC12 represent clusters of identical/highly similar (dissimilarity < 4 bands) REP profiles

Clusters obtained by the Pearson product-moment correlation coefficient are shown in a dendrogram (Fig. 3.20).

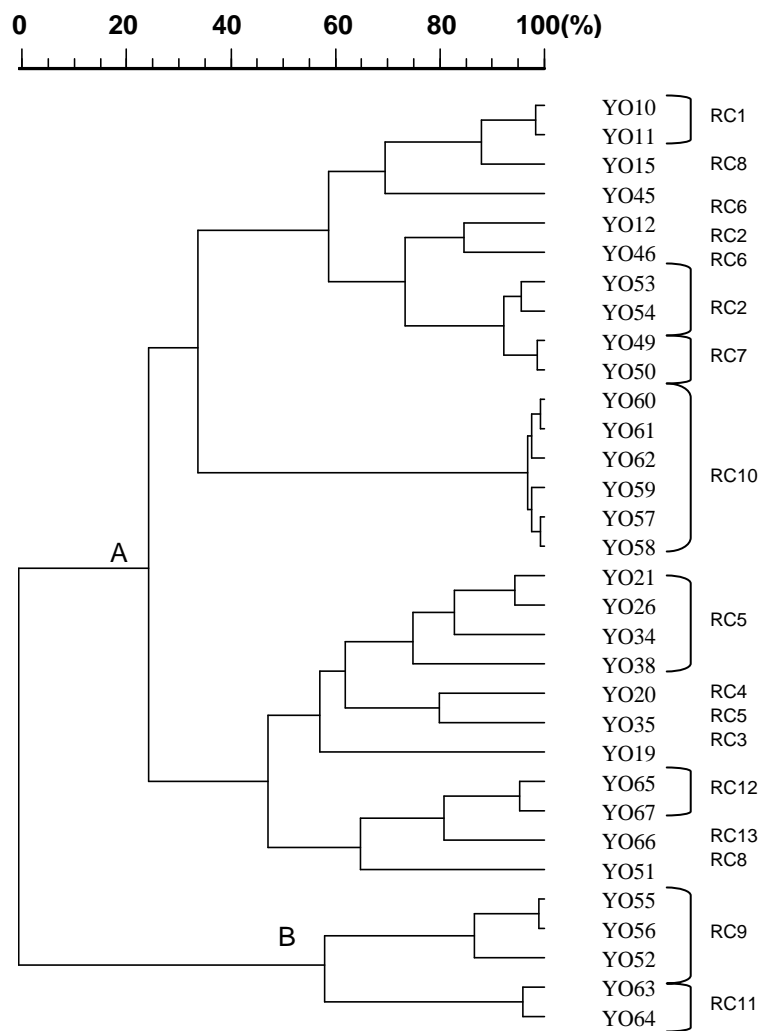


FIG. 3.20. Dendrogram of the cluster analysis of REP profiles of *Flavobacterium* spp. isolates following DNA amplification using the REP primer set REP1 and REP2.

With primer set REP1 and REP2, a total number of 30 clusters were established, at a maximum similarity level of 99.5%. The collection of isolates was divided into 2 major clusters (A and B) at similarity level of 0% (Fig. 3.20). Clusters A and B were further subdivided into 25 and 4 clusters, respectively, comprising of isolates which had also been clustered visually. Although none of the isolates were clustered at 100% similarity, identical patterns were observed during visual analysis. Isolates sharing identical REP profiles, i.e., YO10-YO11 (RC1) clustered at 98% similarity, YO21-YO26 (RC5) clustered at > 90% similarity, YO53-YO54 (RC2) clustered at > 95% similarity, YO55-YO56 (RC9) clustered at 99% similarity, YO57-YO62 (RC10) clustered at 96% similarity, YO63-YO64 (RC11) clustered at 95% similarity, and YO65 + YO67 (RC12) clustered at > 95% similarity, respectively (Fig. 3.20). As observed in visual analysis, isolates YO19, YO51 and YO66 formed single clusters. Due to differences in band intensities and gel alignment, clustering in GelCompar appeared to group isolates differently compared to visual clusters.

3.3.6 Plasmid Profiling Analysis

Single plasmids, ranging from approximately 2.8 kb to 3.5 kb in size, were detectable in plasmid profiles of 90.6% of *Flavobacterium* spp. isolates studied (Fig. 3.21).



FIG. 3.21. Agarose gel electrophoresis of plasmids from *Flavobacterium* spp. isolates. Lane1: pUCBM21 (3 kb), and lanes 2-5: isolates YO10, YO45, and YO49, respectively.

A plasmid of approximately 2.8 kb in size was observed in plasmid profiles of isolates YO10 (Fig. 3.21, Lane 2), YO11, YO12, YO15, YO19, YO20, YO21, YO26, YO34, YO35 and YO38, respectively. A plasmid of approximately 3 kb in size, was observed in plasmid profiles of isolates YO45 (Fig. 3.21, Lane 3), YO46, YO57, YO58, YO59, YO60, YO61, YO63, YO64 and YO66 respectively. A plasmid of approximately 3.5 kb in size, was observed in plasmid profiles of isolates YO49 (Fig. 3.21, Lane 4), YO50 YO51, YO52, YO53, YO54, YO55 and YO56, respectively. No plasmids were detectable in plasmid profiles of isolates YO62, YO65 and YO67 using the modified Birnboim and Doly method (1979). Since only single plasmids were observed in plasmid profiles of all the isolates, plasmid profiling appeared to be of limited use for the discrimination of isolates.

3.3.7 WCP Analysis

Four different protein profiles, forming four prominent clusters, were obtained following visual analysis of WCP profiles of the *Flavobacterium* spp. isolates (Figs. 3.22-3.25). WCP profiles A1, A2, B and C consisted of 29, 30, 35 and 24 bands, respectively, ranging in size from approximately 162 kDa to 24 kDa (Table 3.5). Isolates YO12-YO15, YO45-YO50 and YO53-YO54 exhibited protein profile A1 (Table 3.5, Figs. 3.22-3.23). Isolates YO57-YO58 and YO60-YO62 exhibited protein profile A2, which was identical to profile A1, except for an extra protein band approximately 96 kDa in size (Table 3.5, Fig. 3.24, Lanes 4-5 and Lanes 7-9). Isolates YO19-YO38, YO52, YO55-YO56, YO59, YO63-YO64 and YO66 all exhibited protein profile B (Table 3.5, Figs. 3.22-3.25), whereas only isolates YO51, YO65 and YO67 exhibited protein profile C. Identical bands shared among the different profiles included proteins with molecular weights of approximately 113 kDa, 100 kDa, 68 kDa, 57 kDa, 55 kDa, 52 kDa, 44 kDa, 38 kDa, 37 kDa, 36.5 kDa, and 35.5 kDa, respectively, although band intensities differed slightly. Protein groups with protein molecular weights of approximately 57 kDa, 55 kDa and 52 kDa, and 37 kDa, 36.5 kDa and 35.5 kDa, respectively,

formed 2 prominent clusters of bands shared by all isolates, although these bands differed among the isolates with respect to band intensity.

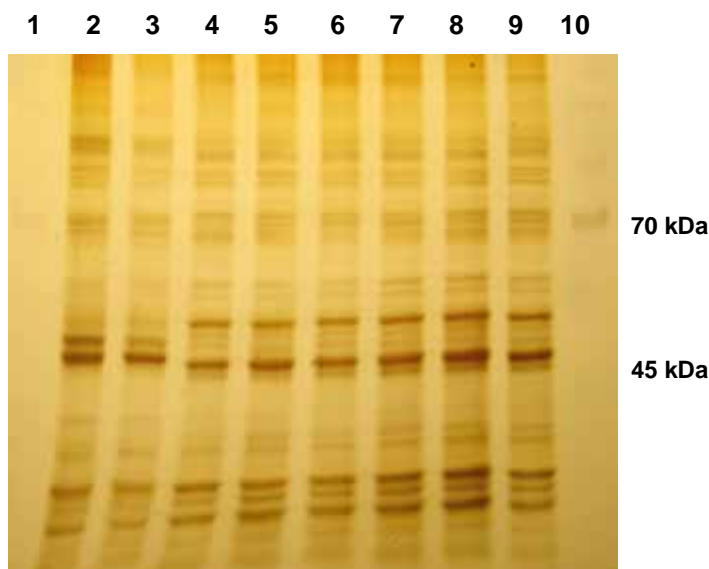


FIG. 3.22. Electrophoretogram displaying whole cell protein profiles of *Flavobacterium* spp. isolates. Lanes 1 and 10: PageRuler™ prestained protein ladder (170/130/100/70/55/45/35/25/15/10 kDa) (Fermentas, Canada), and lanes 2-9: isolates YO12, YO15, YO19, YO20, YO21, YO26, YO34 and YO35, respectively.

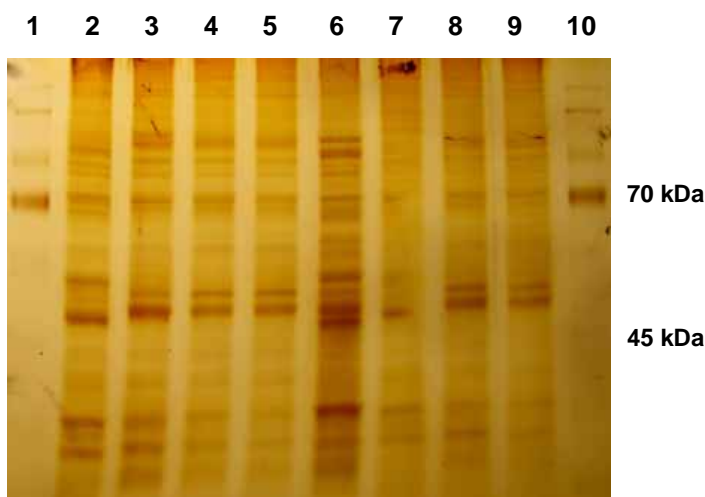


FIG. 3.23. Electrophoretogram displaying whole cell protein profiles of *Flavobacterium* spp. isolates. Lanes 1 and 10: PageRuler™ prestained protein ladder (170/130/100/70/55/45/35/25/15/10 kDa) (Fermentas, Canada), and lanes 2-9: isolates YO38, YO45, YO49, YO50, YO51, YO52, YO53 and YO54, respectively.

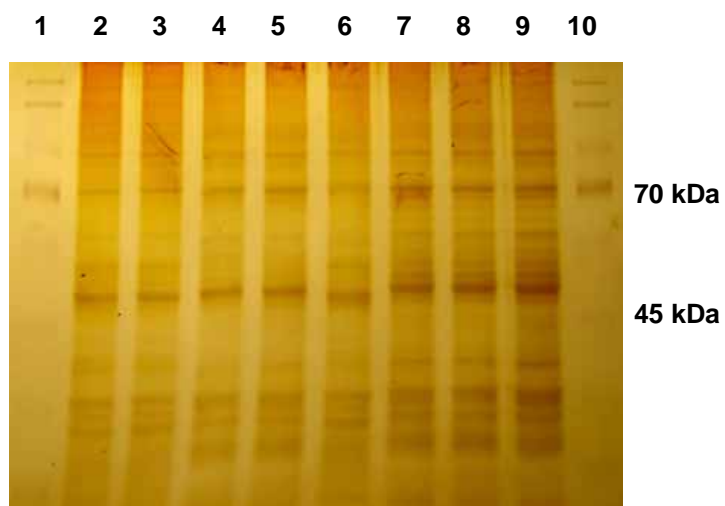


FIG. 3.24. Electrophoretogram displaying whole cell protein profiles of *Flavobacterium* spp. isolates. Lanes 1 and 10: PageRuler™ prestained protein ladder (170/130/100/70/55/45/35/25/15/10 kDa) (Fermentas, Canada), and lanes 2-9: isolates YO55, YO56, YO57, YO58, YO59, YO60, YO61 and YO62, respectively.

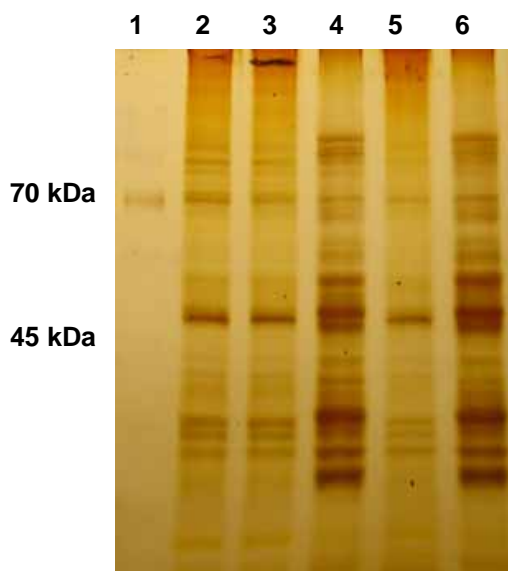


FIG. 3.25. Electrophoretogram displaying whole cell protein profiles of *Flavobacterium* spp. isolates. Lane 1: PageRuler™ prestained protein ladder (170/130/100/70/55/45/35/25/15/10 kDa) (Fermentas, Canada); and lanes 2-6: isolates YO63, YO64, YO65, YO66 and YO67, respectively.

TABLE 3.5. Whole cell protein profiles and protein molecular weight of *Flavobacterium* spp. isolates.

Profile	A1*	A2†	B‡	C§
No. Of fragments	29	30	36	25
Sizes (kDa)	162	162	162	
			160	
			150	
	145	145		
			140	
			132	
	130	130		
	128	128		
			126	
			124	
	120	120	120	
	115	115		
	113	113	113	113
			110	110
				107
	100	100	100	100
		96		
			95	
	90	90		
			88	88
	85	85		
			80	80
				78
			76	
	74	74		
				73
	70	70	70	
	68	68	68	68
			67	
				66.5
			66	
	65	65		65
	60	60	60	
	57	57	57	57
	55	55	55	55
	52	52	52	52
	48	48	48	
				47.5
			47	
	46	46		
			45.5	
	44	44	44	44
			42	
	38	38	38	38
				37.5
	37	37	37	37
	36.5	36.5	36.5	36.5
	35.5	35.5	35.5	35.5
			35.2	35.2
	35	35		
			31	31
	30	30		
	27	27		27
			25	
	24	24		

* Whole cell protein profile A1: isolates YO12, YO15, YO45, YO46, YO49, YO50, YO53 and YO54

† Whole cell protein profile A2: isolates YO57, YO58, YO60, YO61 and YO62

‡ Whole cell protein profile B: isolates YO19, YO20, YO21, YO26, YO34, YO35, YO38, YO52, YO55, YO56, YO59, YO63, YO64 and YO66

§ Whole cell protein profile C: isolates YO51, YO65 and YO67

Clusters obtained by the Pearson product-moment correlation coefficient are shown in a dendrogram (Fig. 3.26).

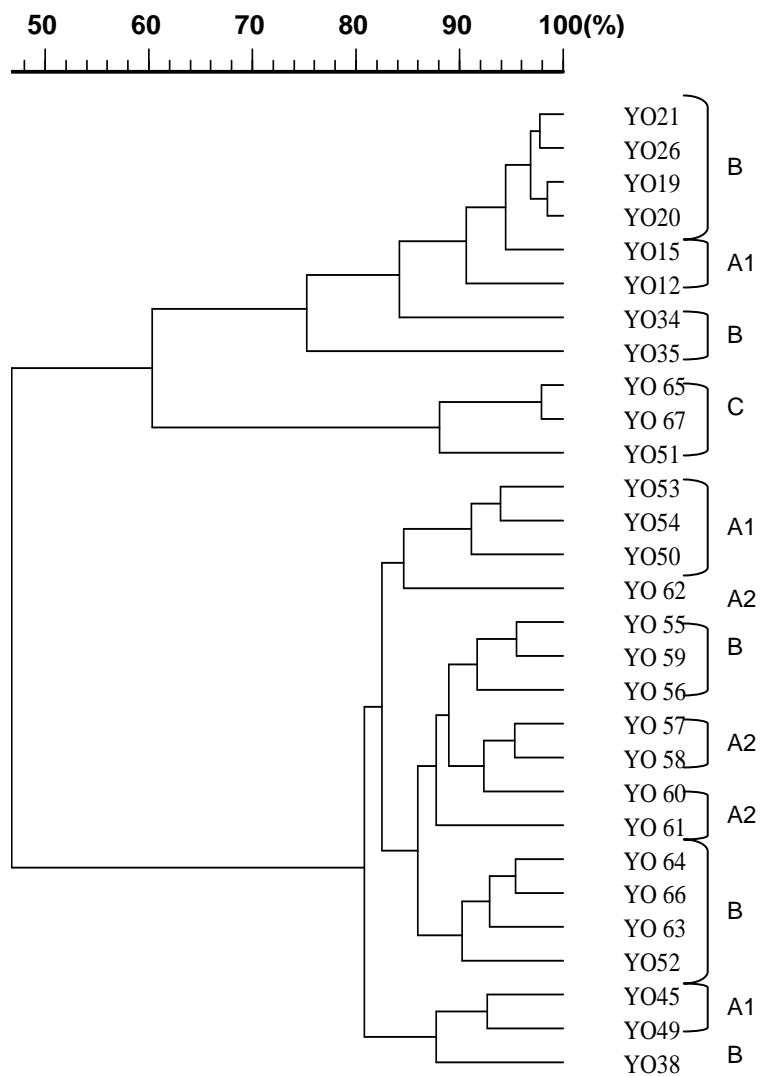


FIG. 3.26. Dendrogram generated by cluster analysis of whole cell protein profiles of the *Flavobacterium* spp. isolates.

A total number of 27 clusters were established, at a maximum similarity level of 98.5%. The collection of isolates was divided into 2 major clusters at 46% similarity. Although none of the isolates were clustered at 100% similarity,

identical profiles were observed during visual analysis. Due to differences in protein band intensities and gel alignment, clustering in GelCompar appeared to group isolates differently compared to visual clusters. Protein profiles A1 and A2 were shown to be almost identical during visual analysis, this has given rise to high similarity displayed by isolates belonging to these clusters in the dendrogram. Isolates YO19-YO35 and isolates YO38, YO52, YO55-YO56, YO59, YO63-YO64 and YO66, all exhibiting protein profile B (Table 3.5), were separated by the two major clusters. Isolates YO51, YO65 and YO67, exhibiting protein profile C and isolates YO57-YO58 and YO60-YO62, exhibiting protein profile A2 (Table 3.5), formed part of one of the two major clusters, although YO62 was grouped with isolates exhibiting protein profile A1. The majority of isolates exhibiting protein profile B were grouped together, only isolates YO34-YO35 and YO38 clustered on their own and shared close similarity to isolates exhibiting protein profile A1. Although certain bands were shared by protein profiles A1 and B, they did not appear similar by visual analysis (Table 3.5, Figs. 3.22-3.25).

3.3.8 OMP Analysis

Eighteen different OMP profiles were established following visual analysis of OMP profiles of the *Flavobacterium* spp. isolates (Figs. 3.27-3.30). OMP profiles consisted of 10-20 bands, ranging in size from approximately 130 kDa to 35 kDa (Table 3.5). An over-expressed OMP, of approximately 35 kDa was present in OMP profiles of all the isolates. Identical bands shared among a large number of different OMP profiles included proteins of approximately 130 kDa, 115 kDa, 75 kDa, 65 kDa, 62 kDa, 55 kDa, 50 kDa, and 37 kDa, respectively, although band intensities differed slightly. Sixteen different OMP clusters (OC1-OC16) were identified visually (Table 3.6). The following clusters of isolates, sharing identical/highly similar OMP fingerprints, were established during visual inspection of OMP profiles: isolates YO20-YO38 (OC4), isolates YO49-YO50

(OC6), isolates YO53-YO54 (OC9), isolates YO55-YO56 (OC10), isolates YO57-58 + YO60 + YO62 (OC11), isolates YO63-YO64 (OC14), and isolates YO65 + YO67 (OC15), respectively (Table 3.6).

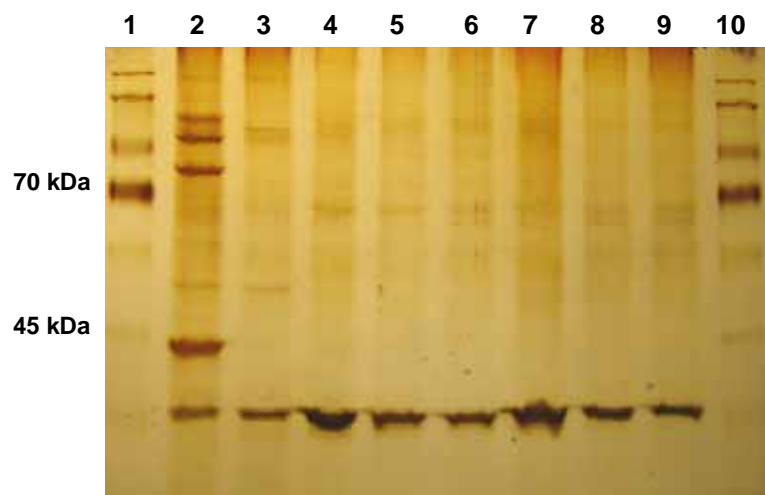


FIG. 3.27. Electrophoretogram displaying outer membrane protein profiles of *Flavobacterium* spp. isolates. Lanes 1 and 10: PageRuler™ prestained protein ladder (170/130/100/70/55/45/35/25/15/10 kDa) (Fermentas, Canada), and lanes 2-9: isolates YO12, YO15, YO19, YO20, YO21, YO26, YO34 and YO35, respectively.

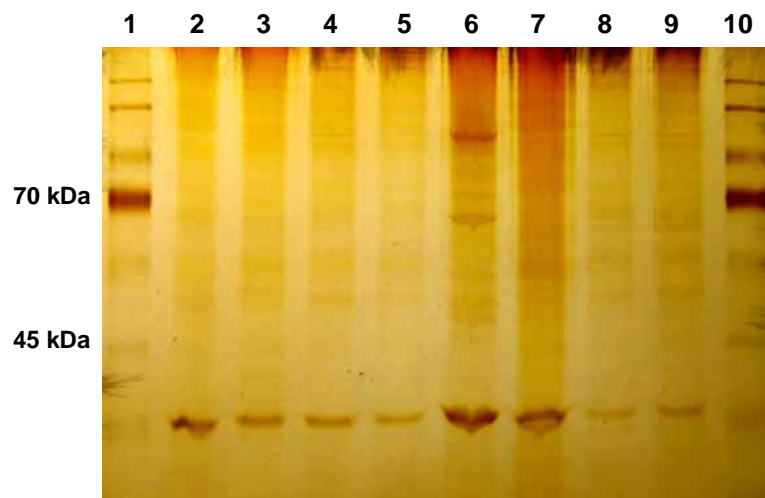


FIG. 3.28. Electrophoretogram displaying outer membrane protein profiles of *Flavobacterium* spp. isolates. Lanes 1 and 10: PageRuler™ prestained protein ladder (170/130/100/70/55/45/35/25/15/10 kDa) (Fermentas, Canada), and lanes 2-9: isolates YO38, YO45, YO49, YO50, YO51, YO52, YO53 and YO54, respectively.

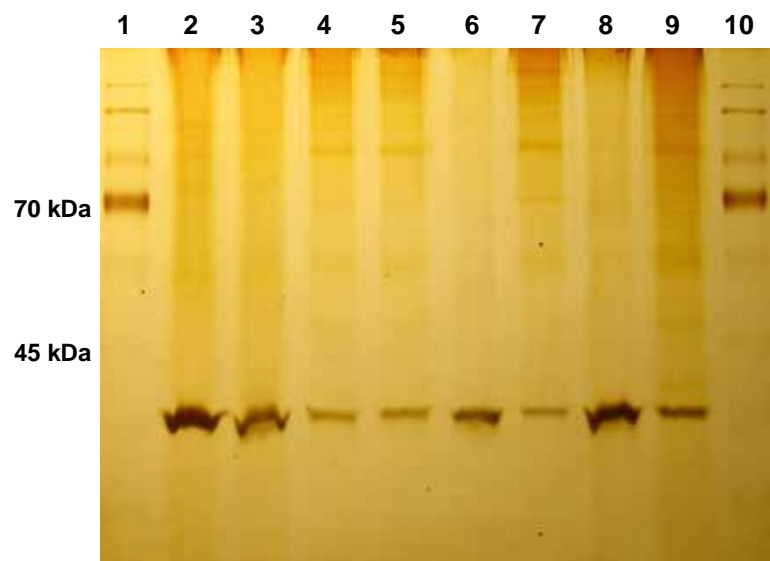


FIG. 3.29. Electrophoretogram displaying outer membrane protein profiles of *Flavobacterium* spp. isolates. Lanes 1 and 10: PageRuler™ prestained protein ladder (170/130/100/70/55/45/35/25/15/10 kDa) (Fermentas, Canada), and lanes 2-9: isolates YO55, YO56, YO57, YO58, YO59, YO60, YO61 and YO62, respectively.

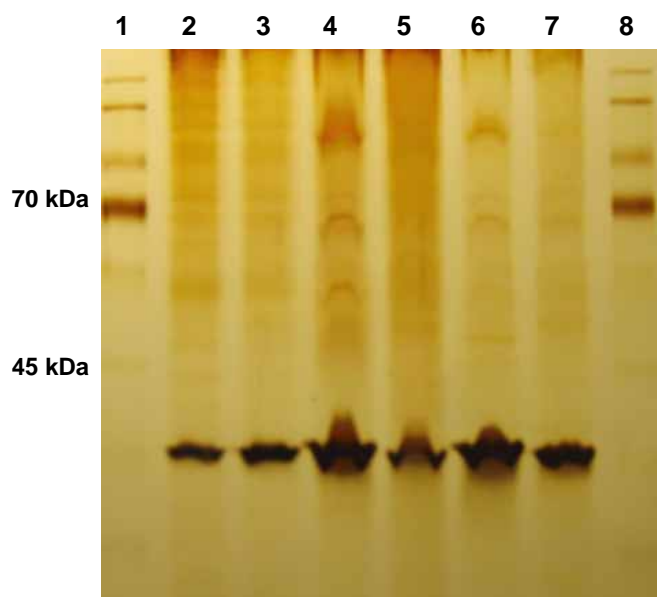


FIG. 3.30. Electrophoretogram displaying outer membrane protein profiles of *Flavobacterium* spp. isolates. Lanes 1 and 8: PageRuler™ prestained protein ladder (170/130/100/70/55/45/35/25/15/10 kDa) (Fermentas, Canada), and lanes 2-7: isolates YO63, YO64, YO65, YO66, and YO67, respectively.

TABLE 3.6. OMP profiles and protein molecular weights of *Flavobacterium* spp. isolates.

[illegible]

*

OC1-OC16 represent clusters of identical/highly similar (dissimilarity < 4 bands) outer membrane protein profiles

Clusters obtained by the Pearson product-moment correlation coefficient are shown in a dendrogram (Fig. 3.31).

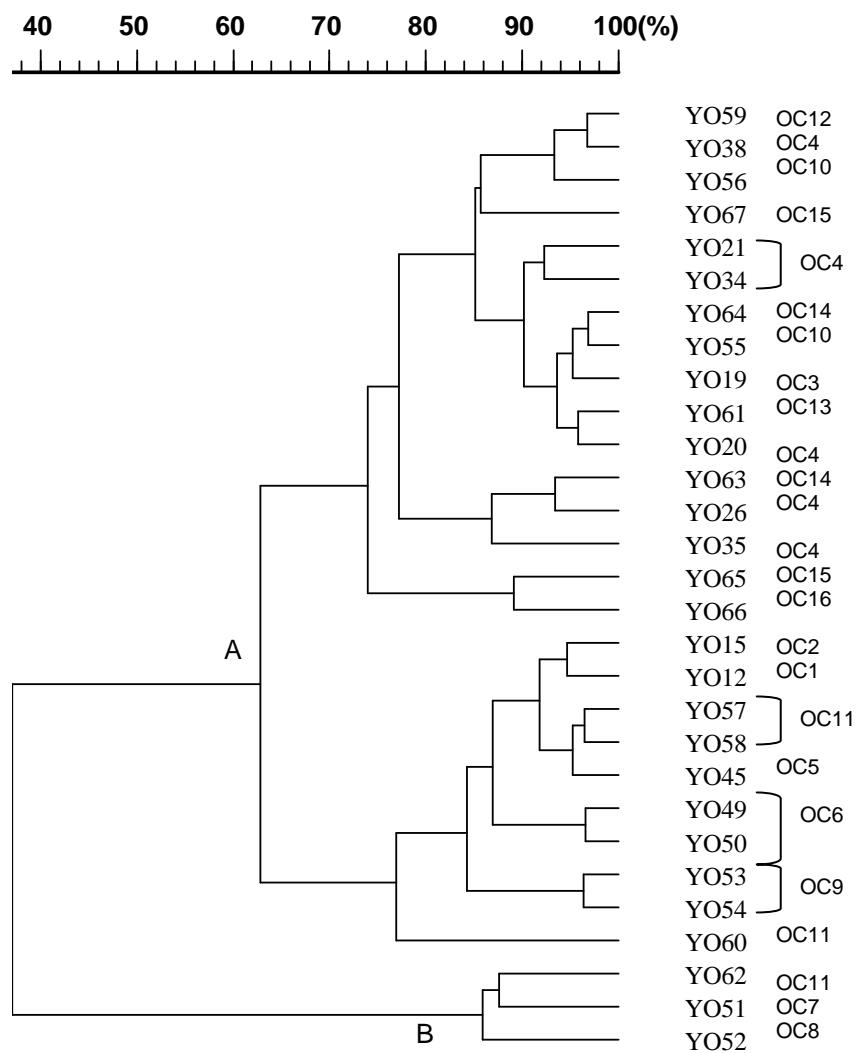


FIG. 3.31. Dendrogram generated by cluster analysis of outer membrane protein profiles of *Flavobacterium* spp. isolates.

A total number of 27 clusters were established, at a maximum similarity level of 96.9%. The collection of isolates was divided into 2 major clusters (A and B) at similarity level of 36% (Fig. 3.31). Clusters A and B were further

subdivided into 25 and 2 clusters, respectively. Although none of the isolates were clustered at 100% similarity, identical patterns were observed during visual analysis. Due to the large amount of different OMP profiles, differences in band intensities and gel alignment, clustering in GelCompar appeared to group isolates differently compared to visual clusters. Of isolates sharing identical OMP profiles only isolates YO21 + YO34 (OC4), YO49-YO50 (OC6), YO53-YO54 (OC9), and YO57-YO58 (OC11) clustered at > 90% similarity (Fig. 3.31, Table 3.6).

3.3.9 Typeability, reproducibility and discriminatory ability of typing methods

Since all the *Flavobacterium* spp. isolates were typeable by PCR-RFLP analysis, RAPD fingerprinting, REP fingerprinting, WCP analysis and OMP analysis, the typeability index was determined at 100% for each respective typing method. Since not all the *Flavobacterium* spp. isolates contained plasmids, the typeability index was calculated to be 91%. Reproducible profiles for the *Flavobacterium* spp. isolates were obtained by all the typing techniques and the reproducibility index was determined at 100% for each respective typing method. Using the Simpson's index of diversity, the discriminatory indices of restriction endonucleases used in 16S rRNA gene PCR-RFLP analysis, RAPD fingerprinting, REP fingerprinting, plasmid profiling, WCP analysis and OMP analysis, respectively, were calculated and are shown in Table 3.7. REP-PCR appeared to be the most discriminatory method for differentiating between *Flavobacterium* spp. isolates. In turn, 16S rRNA gene PCR-RFLP analysis using various restriction endonucleases appeared to be the least discriminatory method.

TABLE 3.7. Discrimination indices for *Flavobacterium* spp. isolates using various typing methods.

Typing method	Number of types	Index (%)
<hr/>		
16S rRNA RFLP:		
<i>AluI</i> (1193 bp)*	1	0
<i>HaeIII</i> (1193 bp)	1	0
<i>HaeIII</i> (1500 bp)†	2	0.3145
<i>HinfI</i> (1193 bp)	1	0
<i>CfoI</i> (1193 bp)	2	0.1210
<i>MspI</i> (1193 bp)	2	0.3871
<i>TaqI</i> (1193 bp)	2	0.3145
RAPD-PCR	16	0.9194
REP-PCR	18	0.9516
Plasmid profiling	3	0.7903
WCP analysis	4	0.6921
OMP analysis	18	0.9458
<hr/>		
* 16S rRNA <i>F. columnare</i> / <i>F. johnsoniae</i> species-specific amplicon		
† 16S rRNA universal amplicon		

3.4 DISCUSSION

The genus *Flavobacterium* has been reclassified by the use of phylogenetic techniques based on the comparison of 16S rRNA gene sequences (Bernardet *et al.*, 2002). Differences in the 16S rRNA gene sequence of up to 5% have been found among strains of some *Flavobacterium* spp., including *F.*

columnare (Bernardet *et al.*, 2002; and Triyanto and Wakabayashi, 1999). In order to restrict the amount of technically demanding investigations, Bernardet *et al.* (2002) recommend newly isolated *Flavobacterium* spp. first be located within the 16S rRNA gene tree.

The 16S rRNA gene sequence analysis of presumptive *Flavobacterium* spp. isolates using both universal and species-specific primers in this study confirmed their identity as being members of the genus *Flavobacterium*. The 16S rRNA species-specific primers Fvp F1 and Fvp R1, originally designed specifically for the amplification of *F. columnare* strains (Bader *et al.*, 2003), was useful for the direct identification of all presumptive *Flavobacterium* spp. isolates. However, 16S rRNA gene sequence analysis showed the *Flavobacterium* spp. isolates were distantly related to *F. columnare* and shared only 92% 16S rRNA gene sequence homology (Appendix 2, Fig. A). Darwish *et al.* (2004) reported the *F. columnare* ATCC 43622 strain, used to design the species-specific primers Fvp F1 and Fvp R1, was in fact a *F. johnsoniae* strain, which was confirmed by biochemical, 16S rRNA gene sequencing and ribotyping analyses. Therefore, these primers were not specific to either *F. columnare* or *F. johnsoniae*, as both species were amplified in the study performed by Bader *et al.* (2003).

Although this PCR technique was not used as a diagnostic tool for the identification of *F. columnare* isolates, it was useful along with biochemical and physiological methods, for the rapid identification of diverse *Flavobacterium* spp. isolates in this study. The lack of amplification with species-specific primers FP1 and FP2 confirmed that none of the *Flavobacterium* spp. isolates was *F. psychrophilum*, however, a fragment in the range of the expected 1088-bp amplicon was amplified from *C. balustinum* NCTC 11212 DNA (Fig. 3.4, Lane 11). *C. balustinum* NCTC 11212 was not included in the study performed by Urdaci *et al.* (1998), however, *C. balustinum* LA 724 used in the study did not produce the 1088-bp product. Urdaci *et al.* (1998) reported that only *F. psychrophilum* isolates produced the 1088-bp amplicon at annealing temperatures $\geq 54^{\circ}\text{C}$. In the present study, numerous amplification products of different sizes were obtained for bacterial strains *C. balustinum* NCTC 11212, *C.*

gleum NCTC 11432, *C. joostei* LMG 18212, *M. odoratus* NCTC 11036 and *S. multivorum* NCTC 11343, respectively, at an annealing temperature of 59°C and PCR conditions described by Urdaci *et al.* (1998) (Fig 3.4). Bacterial species previously classified as *Flavobacterium* spp. have been reclassified to form the genus *Chryseobacterium* during recent amendments (Bernardet and Bowman, 2005; Bernardet *et al.*, 2005; Bernardet *et al.*, 1996; and Bernardet *et al.*, 2002). These genera are closely related and a certain degree of 16S rRNA gene sequence homology between *C. balustinum* NCTC 11212, and other closely related species, and *F. psychrophilum* exists.

This is the first reported isolation and identification of *Flavobacterium* spp. from diseased fish in South Africa. 16S rRNA gene sequence analysis showed that all nine selected *Flavobacterium* spp. isolates shared 16S rRNA gene sequence homology $\geq 97\%$ with each other and the *F. johnsoniae* (AY753067 - Welker *et al.*, 2005) and rainbow trout *Flavobacterium* spp. (AY374109 - Huber *et al.*, 2004) sequences, respectively (Fig 3.2).

Isolates YO10, YO11 and YO15 shared identical 16S rRNA gene sequences (100% sequence similarity) and showed 98% sequence homology with *F. johnsoniae* and rainbow trout *Flavobacterium* spp. (AF336355 - Bohnert *et al.*, 2001) sequences (Fig. 3.2). These isolates were all isolated during the year 2003 from diseased rainbow trout in Franschhoek (Table 2.1) and showed similar biochemical and physiological characteristics, in addition to sharing the typical hazy colony morphology type (Chapter 2, Table 2.2). Isolates YO19, YO34 and YO64 also shared high sequence homology, clustering at 99% similarity (Fig. 3.2), and also showed similar biochemical and physiological characteristics, sharing the smooth colony morphology type and weak proteolytic activity for casein (Chapter 2, Table 2.2). However, these isolates were isolated from different geographical regions and fish host species, at different dates (Chapter 2, Table 2.1). Isolates YO51 and YO60, respectively, clustered on their own, sharing 98% similarity with each other and with the rainbow trout intestinal *Flavobacterium* spp. sequence (AY374109 - Huber *et al.*, 2004) (Fig. 3.2). The biochemical and physiological characteristics of these isolates were distinct from

each other, with isolate YO51 showing the smooth colony morphology and strong gelatin hydrolysis, while isolate YO60 showed the hazy colony morphology type and weak gelatin hydrolysis (Chapter 2, Table 2.1). Additionally, ten isolates, YO10, YO11, YO12, YO15, YO19, YO21, YO26, YO34, YO38 and YO45, were sent to Intervet Norbio Singapour for more thorough phenotypic and biochemical testing (data not shown). These 10 isolates were divided into 2 major clusters, cluster 1 consisting of isolates YO10, YO11, YO12, YO15 + YO45, and cluster 2 consisting of YO19, YO21, YO26, YO34 + YO38, respectively, biochemically, with cluster 1 showing similarity to *F. johnsoniae* and *F. hydatidis* profiles, while cluster 2 was closer to the genus *Sphingobacterium*. A close relationship between 16S rRNA gene sequences of *Flavobacterium* spp. isolates YO10, YO11 and YO15 and *F. johnsoniae* and *F. hydatidis* was indeed observed (Fig. 3.3). However, from 16S rRNA gene data, isolates YO19, YO21, YO34 and YO38 clearly belonged to the genus *Flavobacterium*, although their biochemical characteristics were similar to members of the genus *Sphingobacterium*. Phylogenetic studies involving sequence analysis of *gyrB* and ISR elements may be used in combination with 16S rRNA gene sequence analysis and DNA relatedness studies as a more thorough polyphasic approach to identify these *Flavobacterium* spp. isolates sharing high sequence similarity to *F. johnsoniae*.

Triyanto and Wakabayashi (1999) established three genomovars for *F. columnare* based on *HaeIII* restriction of universal 16S rRNA PCR amplimers. These genomovars have been confirmed by other studies involving *F. columnare* (Arias *et al.*, 2004; and Michel *et al.*, 2002). In the present study, *HaeIII* RFLP profiles of 16S rRNA amplicons of *Flavobacterium* spp. isolates, obtained using universal primers 16S-F and 16S-R, did not correspond to *F. columnare* genomovars described by Triyanto and Wakabayashi (1999), although several similar sized fragments, 300 bp, 280 bp and 120 bp, respectively, were observed (Table 3.2, Fig. 3.7). The *E. coli* numbering positions of the universal primers 20F and 1500R used by Triyanto and Wakabayashi (1999) were 8-27 and 1510-1492, respectively, whereas the *E. coli* numbering positions of the universal primers used in this study were 10-27 and 1507-1485 (Heyndrickx *et al.*, 1996),

respectively. Therefore, the amplified regions were almost identical and would not have affected the resulting restriction fragment sizes to the extent of the differences observed in PCR-RFLP profiles. However, the distant relationship between 16S rRNA gene sequences of the *Flavobacterium* spp. study isolates and that of *F. columnare* displayed by 16S rRNA gene sequence analysis (Appendix A, Fig. 1) explains the differences observed in *HaeIII* profiles of the *Flavobacterium* spp. study isolates compared to that of the *F. columnare* genomovars (Triyanto and Wakabayashi, 1999). Thus it was clear that none of the *Flavobacterium* spp. isolates in the present study were closely related to *F. columnare*.

All the *Flavobacterium* spp. isolates were typeable by 16S rRNA PCR-RFLP analysis using restriction endonucleases *AluI*, *CfoI*, *HaeIII*, *HinfI*, *MspI*, and *TaqI*, with a typeability index of 100%. Although very few patterns were generated, the PCR-RFLP patterns were highly reproducible, with a reproducibility index of 100%. However, this technique showed poor discrimination of the *Flavobacterium* spp. isolates, with restriction endonucleases *AluI*, *HaeIII* and *HinfI*, used in restriction of the 1193 bp species-specific amplicon, showing no discrimination (Table 3.7).

Groups of *Flavobacterium* spp. isolates were clustered to a certain degree by 16S rRNA PCR-RFLP analysis using restriction endonucleases *CfoI*, *HaeIII*, *MspI*, and *TaqI* (Table 3.1). Although, PCR-RFLP analysis of *F. columnare* and *F. psychrophilum* strains has been described as a rapid and effective method for genotyping and differentiating among species and strains related to this genus (Izumi *et al.*, 2000), 16S rRNA gene PCR-RFLP patterns generated using a variety of restriction endonucleases were found to be rather homogeneous with poor discrimination among the *Flavobacterium* spp. isolates tested in the present study.

The UBC 214 primer used for RAPD fingerprinting of the *Flavobacterium* spp. isolates in this study generated 16 different RAPD fingerprints for all isolates (Table 3.3). The polymorphisms observed in the RAPD profiles generated by amplification of *Flavobacterium* spp. isolates DNA using primer UBC 214

indicated a high degree of genetic heterogeneity between the isolates. A number of prominent bands, approximately 3170 bp, 1750 bp, 1030 bp, 950 bp, 870 bp, 770 bp, 630 bp, 470 bp and 410 bp in size, respectively, were amplified from the majority of the *Flavobacterium* spp. isolates (Table 3.3). The 870 bp fragment was present in all the RAPD fingerprints, however, it varied in intensity from isolate to isolate and from profile to profile (Figs. 3.13-3.15). This band also formed part of a prominent cluster of bands, approximately 1030 bp, 950 bp and 870 bp, observed in 72% of the profiles (Table 3.3). The use of genetic markers as a means of identification greatly depends on the frequency at which the characteristic markers are expressed within a given population (Bassam *et al.*, 1992). The prominent cluster of bands, or the 870 bp fragment on its own, displayed in the RAPD fingerprints of *Flavobacterium* spp. isolates may potentially be useful as genetic markers specific to these *Flavobacterium* spp. strains or the genus *Flavobacterium* as a whole.

Although in most cases, visual groupings of profiles corresponded to clusters obtained following GelCompar analysis, it is possible to explain the discrepancy between the two methods. Variability in band intensities, the presence of primary, secondary and tertiary amplicons (Bassam *et al.*, 1992) and poor gel alignment due to slight differences in electrophoretic conditions affected the clustering of RAPD profiles as observed in Fig. 3.16. Hence, visual analysis of documented RAPD profiles appear superior. Band intensity was disregarded in favour of presence/absence of bands and this allowed a more accurate assessment of clusters sharing similar or identical RAPD profiles.

A number of differential PCR-based typing methods have been performed on *Flavobacterium* species (Bernardet and Bowman, 2005; Chakroun *et al.*, 1997; and Crump *et al.*, 2001). RAPD-PCR has been successfully used in differentiating among *Flavobacterium* spp. at both species and strain level (Chakroun *et al.*, 1997; and Crump *et al.*, 2001). Results obtained have been shown to be dependent on the primers used, which for certain primers either reveal a clear correlation between RAPD profiles and fish species from which strains have been isolated or reveal unique RAPD profiles for all *F.*

psychrophilum isolates (Chakroun *et al.*, 1997). However, no correlation between RAPD profiles and geographic origin of *Flavobacterium* spp. has been documented (Bernardet *et al.*, 2005; Chakroun *et al.*, 1997). The RAPD technique used in the current study was able to discriminate between different *Flavobacterium* spp. isolates obtained from both different fish species as well as from different geographical regions in South Africa. In addition to being highly reproducible, a discrimination index of 91.93% was obtained for the RAPD technique used in this study, which fell within the desirable discrimination index of $\geq 90\%$.

REP-PCR fingerprinting has recently been used to investigate the genomic diversity among *Flavobacterium* spp. isolated from polar regions (Bernardet and Bowman, 2005). According to Bernardet and Bowman (2005), identical REP profiles were obtained for closely related strains and may be beneficial in differentiating among *Flavobacterium* spp. or for the use of intraspecific typing.

As with RAPD-PCR, REP-PCR fingerprinting of the *Flavobacterium* spp. isolates in this study generated reproducible REP fingerprints for all isolates, however, a total number of 18 profiles were produced, suggesting a greater degree of genetic heterogeneity among isolates (Table 3.4). Two prominent bands, approximately 3310 bp and 590 bp in size were observed in 7 and 14 different REP profiles, respectively. In contrast to RAPD fingerprinting, no band/s common to all the REP fingerprints were observed. As with RAPD profiles, primary, secondary and tertiary bands were also present in the REP fingerprints. Several bands present in the REP fingerprints varied in intensity from isolate to isolate and from profile to profile (Figs. 3.17-3.19). Although in most cases, visual groupings of REP profiles corresponded to clusters obtained following GelCompar analysis, it is possible to explain the discrepancy. Variability in band intensities, the presence of primary, secondary and tertiary amplicons (Bassam *et al.*, 1992) and poor gel alignment due to slight differences in electrophoretic conditions affected the clustering of REP profiles as observed in Fig. 3.20. As with RAPD analysis, visual analysis of documented REP profiles appear superior.

Similarly, band intensity was disregarded in favour of presence/absence of bands and this allowed a more accurate assessment of clusters sharing similar or identical REP profiles.

Both the RAPD and REP techniques used in this study were shown to discriminate between the different *Flavobacterium* spp. isolates used in this study. A discrimination index of 95.16% was obtained for the REP technique used in this study. This also fell within the desirable discrimination index of $\geq 90\%$. A good correlation was obtained between the results of RAPD and REP fingerprinting. Isolates which clustered visually or via GelCompar analysis of RAPD profiles also clustered on examination of their REP profiles, thus consistent grouping of isolates belonging to specific clusters was observed. No correlation could be established between fish host species and geographical origin and REP and/or RAPD profiles of the *Flavobacterium* spp. isolates.

The use of ERIC-PCR has been reported for studying the genetic diversity and characterisation of aquatic pathogenic bacteria, such as *Yersinia ruckeri* (Coquet *et al.*, 2002a) and *Aeromonas* spp. (Szczuka and Kaznowski, 2004). Very few bands were obtained with the ERIC-PCR technique used in this study, suggesting that the ERIC elements may not be widely distributed in the genomic DNA of the *Flavobacterium* spp. isolates under investigation.

Madsen and Dalsgaard (2000) have found great homogeneity among plasmid profiles of *F. psychrophilum* and suggested a possible correlation between ribotypes, serotypes and plasmid profiles, while differences in plasmid profiles among *F. psychrophilum* strains have also been described to be related to difference in pathogenicity (Lorenzen *et al.*, 1997). Three different plasmid profiles obtained in this study constituted of single plasmids, approximately 2.8 kb, 3 kb and 3.5 kb in size (Fig. 3.21), respectively, while in a fourth group plasmids were not detected using the modified Birnboim and Doly method (1979). The absence of a plasmid, the rough method used to extract plasmids and/or the presence of extremely large plasmids, either improperly extracted or impaired mobility of plasmids in gel electrophoresis, may in part explain plasmid absence in this group of isolates. Plasmids may also be lost or gained over time,

which may have affected plasmid profiles of isolates YO62, YO65 and YO67, especially in the absence of selective pressures.

The plasmid profiles were very homogeneous with poor discrimination among *Flavobacterium* spp. isolates, which was evident in the discrimination index of 79.03%. This is in accordance with Chakroun *et al.* (1998) and Madsen and Dalsgaard (2000) who reported plasmid profiles of *F. psychrophilum* were very homogeneous and plasmid profiling for epizootiological investigations was not recommended. Furthermore, the isolates grouped by plasmid profiling did not correlate with any of the clusters obtained by 16S rRNA gene PCR-RFLP analysis, RAPD-PCR fingerprinting or REP-PCR fingerprinting. No correlation between plasmids, biochemical and physiological characteristics of *Flavobacterium* spp. isolates was observed.

Flavobacterium spp., such as *F. columnare* and *F. psychrophilum*, have been identified according to specific protein profiles, whereas other species, including *F. branchiophilum*, *F. johnsoniae* and *F. succinicans*, display great heterogeneity in their protein profiles (Bernardet and Bowman, 2005; Bernardet *et al.*, 1996). Cipriano and Teska (1994) showed WCP profiles of *F. columnare* and *F. psychrophilum* remained consistent regardless of the growth medium used. However, standardized SDS-PAGE conditions are recommended for comparative analysis of *Flavobacterium* spp. protein profiles.

Four distinct WCP profiles were obtained by SDS-PAGE of whole cell lysates of the *Flavobacterium* spp. isolates in this study (Table 3.5). Protein bands varied in molecular weight from approximately 162 kDa to 24 kDa, with prominent bands approximately 113 kDa, 100 kDa, 68 kDa, 57 kDa, 55 kDa, 52 kDa, 44 kDa, 38 kDa, 37 kDa, 36.5 kDa and 35.5 kDa in size, shared by all four profiles. Bands of approximately 57 kDa, 55 kDa and 52 kDa, and 37 kDa, 36.5 kDa and 35.5 kDa, respectively, formed two prominent clusters present in all four profiles. These identical bands differed slightly in intensity, which may be attributed to variation in expression levels among isolates or may be a concentration effect.

As mentioned previously, differences in groups of isolates observed in the dendrogram (Fig. 3.26) compared to clusters obtained by visual analysis may be due to differences in band intensities, resulting from both variation in levels of protein expression and the concentration effect, and slight differences in SDS-PAGE, leading to poor gel alignment.

Although high similarity in WCP profiles of certain *Flavobacterium* spp. have been correlated with high DNA homology, the only WCP profile obtained in this study which correlated with clusters sharing high 16S rRNA gene homology, was WCP profile B. Isolates YO19, YO34 and YO64, exhibiting this WCP profile, showed 99% similarity in 16S rRNA gene sequence analysis. These isolates possibly represent a specific *Flavobacterium* spp. strain. Clustering of the isolates according to the different WCP profiles correlated with clusters obtained in RAPD and REP fingerprinting to a certain degree. Isolates YO12-YO15, YO49-YO50 and YO53-YO54, consistently clustered by RAPD and REP fingerprinting, all exhibited WCP profile A1 (Table 3.5). Isolates YO19-YO38, isolates YO52 and YO55-YO56, and isolates YO63-YO64, respectively, exhibiting WCP profile B, were similarly clustered by both RAPD and REP fingerprinting, however, the RAPD and REP fingerprints differed among the clusters. With the exception of isolate YO59, exhibiting WCP profile B, isolates YO57-YO58 and YO60-YO62, exhibiting WCP profile A2, shared identical to highly similar profiles by RAPD and REP fingerprinting. Isolates YO51, YO65 and YO67, exhibiting WCP profile C, also clustered by RAPD fingerprinting. WCP profiles could not be distinctly correlated with any of the biochemical or physiological characteristics of the *Flavobacterium* spp. isolates, however, all the isolates exhibiting WCP profile A1 showed strong proteolytic activity for casein. No correlation between WCP profiles and fish host species or geographical origin could be established.

Crump *et al.* (2001) characterized cellular antigens and surface antigens of *F. psychrophilum* strains by Western blot analysis and biotin labeling, respectively, of whole cell lysates. Cellular antigens ranging in size from approximately 16 kDa to 80 kDa were observed in characteristically similar

patterns. Surface antigens included a major antigen approximately 16 kDa in size, as well as other bands approximately 22, 24, 35, 47, 60, 74, and 100 kDa in size. Many bands ranging in size from 25 kDa to ≥ 80 kDa were observed in WCP profiles of the *Flavobacterium* spp. isolates, however, proteins of similar or identical in size to many of the surface antigens detected by Crump *et al.* (2001) were present in the different WCP profiles of the *Flavobacterium* spp. isolates.

F. psychrophilum extracellular metalloproteases Fpp1 (~55 kDa) and Fpp2 (~62 kDa) were purified from the supernatant of centrifuged cells by Secades *et al.* (2001) and Secades *et al.* (2003), respectively. Although these proteins have an extracellular function, a certain concentration may still be isolated from the cell cytosol or periplasmic space, before protein export has taken place. A protein of identical size to Fpp1 was detected in all the WCP profiles of the *Flavobacterium* spp. isolates, however, clear differences in proteolytic activity for casein was observed among the isolates, with some showing weak proteolytic activity (Chapter 2, Table 2.2). Additionally, differences in protein concentrations of the ~55 kDa protein observed in the WCP profiles did not correlate with differences in proteolytic activity for casein, and band intensity was higher in isolates showing weak proteolytic activity. Although a protein similar in size to Fpp2 was present in WCP profile B, the majority of these isolates showed weak proteolytic activity for casein even though Fpp2 is also known to degrade casein. Metalloproteases Fpp1 and Fpp2 may be specific to *F. psychrophilum* (Secades *et al.*, 2001; and Secades *et al.*, 2003). Proteolytic activity displayed by the *Flavobacterium* spp. isolates in the present study may be related to unidentified proteases and requires further investigation.

All the *Flavobacterium* spp. isolates were typeable with WCP profiling and the WCP profiles were highly reproducible. Although similarities were found with clusters obtained by RAPD and REP analysis, WCP analysis was not discriminatory enough to be used alone (discriminatory index of 69.21%), and should be used in conjunction with molecular typing methods or other chemotaxonomic typing methods.

OMP profiling may not necessarily be used as a typing tool, but may reveal important information about bacterial outer membrane constituents, involved in activities such as bacterial aggregation, adherence, and the efflux and influx of chemicals. Cell envelope proteins of flavobacteria are regarded as important factors in pathogenesis and constitute essential components for possible vaccines against *F. psychrophilum* infection (Merle *et al.*, 2003). Bacterial outer membrane constituents, including outer membrane proteins and porins, have also been linked to biofilm formation and consequently virulence of pathogenic bacteria (Bell, 2001; Donlan, 2002; Stickler, 1999; and Webb *et al.*, 2003). Therefore, studying OMP profiles can also reveal important information about the biofilm forming components of *Flavobacterium* spp. and a possible correlation with virulence.

Eighteen different OMP profiles were obtained by OMP analysis of the *Flavobacterium* spp. isolates used in this study. OMPs varied in size from 130 kDa to 35 kDa. Nine prominent OMPs, approximately 130 kDa, 115 kDa, 75 kDa, 65 kDa, 62 kDa, 55 kDa, 50 kDa, 46 kDa, 37 kDa and 35 kDa in size, were in many of the profiles, with the ~35 kDa OMP being present in all the profiles. This ~35 kDa OMP was expressed at a much higher level by all the isolates and probably constituted a major OMP. This major OMP differed slightly in intensity among the isolates, which seemed profile-specific. The OMP profile of isolate YO12 exhibited 3 additional over-expressed bands, of approximately 113 kDa, 107 kDa and 50 kDa in size (Fig. 3.27). Although these OMPs were present in other profiles, they were not expressed at the same level and appeared much less intense (Table 3.6, Figs. 3.27-3.30). The majority of the profiles shared two prominent sets of OMPs, of approximately 65 kDa + 62 kDa, and 37 kDa + 35 kDa in size, respectively. Many of the isolates clustered by OMP analysis correlated with the clusters obtained by RAPD and REP fingerprinting, suggesting clonal origins. Dominant clonal groups of *Flavobacterium* spp. study isolates obtained from rainbow trout and eel, included *Flavobacterium* spp. isolates YO21-YO38 and *Flavobacterium* spp. isolates YO57-YO58 + YO60-YO62, respectively (Chapter 2, Table 2.1, and Tables 3.3, 3.4 and 3.6). The fish

species these *Flavobacterium* spp. study isolates were obtained from, may serve as dominant host species for these respective *Flavobacterium* spp. clones. OMP profiles showed very low homology with WCP profiles of corresponding isolates. However, prominent clusters obtained by OMP analysis, which corresponded to clusters obtained by WCP analysis included isolates YO20-YO38, isolates YO49-YO50 and YO53-YO54, isolates YO57-YO58 and YO60 and YO62, isolates YO63-YO64, and isolates YO65 and YO67. Isolate YO19 exhibited a unique OMP profile, although many common bands were shared with the clustered isolates YO20-YO38. Although the OMP profiles of isolates YO57-YO62 shared many common bands, they were not found to be highly similar and differences in levels of OMP expression was also observed (Fig. 3.29)

Many isolates sharing identical or highly similar OMP profiles were not clustered. Differences in expression level of OMPs, the concentration effect, and slight differences in SDS-PAGE conditions, leading to poor gel alignment, may explain the differences in groups obtained by GelCompar analysis compared to clusters established during visual analysis. As with all the other typing methods used in this study, visual analysis of resulting fingerprints was superior.

Overall, good discrimination of the *Flavobacterium* spp. isolates was obtained by OMP profiling, and the discrimination index for OMP analysis was calculated at 94.58%, which is close to the desired discrimination index of $\geq 90\%$. As with WCP profiles, the profiles generated by OMP analysis were also highly reproducible under the standard conditions used in this study.

Many common bands, approximately 130 kDa, 128 kDa, 124 kDa, 120 kDa, 115 kDa, 113 kDa, 107 kDa, 100 kDa, 90 kDa, 85 kDa, 80 kDa, 70 kDa, 68 kDa, 65 kDa, 63 kDa, 60 kDa, 57 kDa, 55 kDa, 52 kDa, 48 kDa, 38 kDa, 37 kDa and 35 kDa in size, were shared between OMP and WCP profiles, however, these bands were not confined to specific clusters and appeared random. Proteins of similar size or molecular mass may co-migrate during SDS-PAGE, producing single bands on electrophoretograms. Additionally, the presence of capsular material, inner membrane obstruction, other outer membrane constituents (phospholipids and LPS), and carbohydrate moieties of many OMPs

complicate their extraction and simple methods used to extract WCPs may not effectively extract OMPs (Benedí and Martínez- Martínez, 2001, and Lugtenberg, 1981). Hence, these common protein bands may not represent identical proteins.

As with WCP analysis, proteins of similar or identical size to many of the surface antigens detected by Crump *et al.* (2001) were present in the OMP profiles, including OMPs with approximate molecular masses of 35 kDa, 60 kDa and 100 kDa. The cell envelope of *F. psychrophilum* contains more than 50 polypeptides, including a major component, P60, with molecular mass of 65 kDa (Merle *et al.*, 2003). An OMP of approximately 65 kDa was present in the majority of the OMP profiles, however, the band appeared of low intensity and may not constitute a major OMP. A protein of the same approximate molecular mass was also evident in WCP profiles A1, A2 and C, however, these profiles did not correspond to the isolates exhibiting the ~65 kDa OMP. Further investigation is needed for identification of OMPs obtained by OMP analysis of the *Flavobacterium* spp. isolates.

OMPs are known to play an integral role in bacterial attachment and subsequent biofilm formation (Donlan, 2002; Gavín *et al.*, 2003, and Stickler, 1999). Further investigation is needed to establish a possible correlation between the presence of these proteins, virulence and/or biofilm formation by *Flavobacterium* spp. The biofilm-forming capabilities of *Flavobacterium* spp. have not been investigated in depth at present. Therefore, in the following chapter, the biofilm-forming capabilities of the *Flavobacterium* spp. isolates were investigated using microtiter-plate adherence assays and flow cell studies, in part, to establish a possible correlation between the biofilm phenotype and specific genotypes/phenotypes displayed by *Flavobacterium* spp. isolates in the present study.

CHAPTER FOUR

CHARACTERISATION OF THE BIOFILM-FORMING CAPABILITY OF *Flavobacterium* spp.

4.1 INTRODUCTION

Microbial colonization of surfaces, usually comprised of multicellular attachments suspended in extracellular polymeric substances, forming a slimy layer is referred to as biofilms (Bell, 2001; Donlan, 2002; Stickler, 1999; and Webb *et al.*, 2003). Bacteria colonize surrounding surfaces to form sessile communities, displaying remarkably different synthetic and metabolic characteristics compared to related cells occurring in the planktonic state (Bell, 2001; Donlan, 2002; and Webb *et al.*, 2003). This has led to a new understanding of biofilm-associated microorganisms and the microbial complexity within biofilms, for it has become increasingly difficult to treat biofilm-associated infections present in both humans and animals (Bell, 2001; Donlan, 2002; and Webb *et al.*, 2003).

Biofilms have been successfully studied and recreated in the laboratory environment by the use of several techniques, such as solid supports suspended in beakers (Coquet *et al.*, 2002a; Critchley *et al.*, 2003; Elvers *et al.*, 1998; Karunasagar *et al.*, 1996; and Wang *et al.*, 2002), microtiter-plate assays (Croxatto *et al.*, 2002; Gavín *et al.*, 2003; O'Toole *et al.*, 1999; and Stepanovic *et al.*, 2000), flow cell studies (Hall-Stoodley and Lappin-Scott, 1998; and Wang *et al.*, 2003), bioreactor studies (Banning *et al.*, 2003; and Bremer *et al.*, 2002) and the use of several molecular techniques (Oppong *et al.*, 2003; and O'Sullivan *et al.*, 2002).

Environmental biofilm samples have been collected by mechanical removal, such as scraping or swabbing (Banning *et al.*, 2003; Coquet *et al.*, 2002a; Critchley *et al.*, 2003; Elvers *et al.*, 1998; Mridula *et al.*, 2003; and O'Sullivan *et al.*, 2002). Microorganisms isolated from a diversity of biofilm environments (water, human or animal infections) have been tested in adherence

and biofilm studies (Bomo *et al.*, 2003; Coquet *et al.*, 2002a; Croxatto *et al.*, 2002; Karunasagar *et al.*, 1996; Mridula *et al.*, 2003; and Wang *et al.*, 2002).

Biofilm complexity has been studied using various cultivation techniques, such as the spread plate technique (Critchley *et al.*, 2003; Elvers *et al.*, 1998; and Mridula *et al.*, 2003). Oppong *et al.* (2003) used the spread plate technique to isolate a number of filamentous microorganisms from paper mill slimes and the 16S rRNA gene sequence of isolate SAI coincidentally showed a 92% homology to *Cytophaga* spp. or *F. columnare*. Mridula *et al.* (2003) studied the water quality, biofilm production of microorganisms and growth of fringe-lipped carp in tanks. Scrapes of biofilm growth and water samples were analyzed by the spread plate technique on various solid media from which plate counts were made after incubation to determine the microbial density.

Molecular techniques have been established to analyze biofilms and the genes involved in biofilm formation (O'Sullivan *et al.*, 2002; and O'Toole *et al.*, 1999). O'Sullivan *et al.* (2002) studied the bacterial diversity in river epilithon, focusing on biofilms formed on stones in aqueous environments. Biofilm growth was scrubbed from stones collected from River Taff and DNA extracted directly from these epilithon samples were used in 16S rRNA PCR amplification. The construction of clone libraries allowed sequencing and slot blot hybridizations with taxon specific 16S rRNA-targeted oligonucleotide probes, which revealed members of the *Cytophaga-Flavobacterium-Bacteroides* phylum, including *Flavobacterium* species.

Microtiter-plate adherence assays have been developed as a rapid method for investigating biofilm-forming abilities of microorganisms. This technique involves investigating bacterial attachment to the surface of multiwell plates. Typically used microtitre-plates consist of PVC or polystyrene, however, polypropylene, polycarbonate plastic or borosilicate glass have also been proven effective (O'Toole *et al.*, 1999). Microtiter-plate adherence assays are useful for investigating large quantities of isolates or bacterial strains for biofilm formation capability and ideal for studying the early and later developmental stages of biofilm formation (O'Toole *et al.*, 1999). Various modifications of this technique

have been described as more organisms are studied (Croxatto *et al.*, 2002; Gavín *et al.*, 2003; O'Toole *et al.*, 1999; and Stepanovic *et al.*, 2000). Stepanovic *et al.* (2000) devised a modified microtiter-plate assay, which has been found to be superior in terms of accuracy and objectivity compared favorably with qualitative data obtained from biofilm formation studies using the tube test. The ability of *Aeromonas* spp. (Gavín *et al.*, 2003) and *V. anguillarum* (Gavín *et al.*, 2003) to form biofilms *in vitro* has been successfully studied with the use of microtiter-plate adherence assays. This technique may be effective for the investigation and characterisation of biofilm formation by *Flavobacterium* spp. in studies investigating large quantities of isolates.

The ability of microorganisms to adhere to solid supports has been investigated using different materials as suitable surfaces for biofilm formation (Coquet *et al.*, 2002a; Critchley *et al.*, 2003; Elvers *et al.*, 1998; Karunasagar *et al.*, 1996; and Wang *et al.*, 2002). Wang *et al.* (2002) studied the biofilm-forming ability of *Vibrio anguillarum*, a fish pathogen, by determining the percentage area coverage due to biofilm-formation on glass slides suspended in inoculated beakers. Similarly, Coquet *et al.* (2002a) investigated the ability of *Yersinia ruckeri* to adhere to wood supports, which were suspended in beakers containing inoculated broth. Strains tested were found to exhibit a strong affinity to the surface of wooden supports, which corresponded with results from previous studies. Similar studies have been performed using copper coupons (Critchley *et al.*, 2003), and PVC surfaces in mixed and single recirculating batch culture systems (Elvers *et al.*, 1998). Karunasagar *et al.* (1996) investigated the ability of *Vibrio harveyi*, a shrimp pathogen, to attach to various materials present in aquaculture settings, viz., concrete slabs, stainless steel and plastic (high density polyethylene) coupons. Biofilms were detected on all three substrates tested indicating the relevance of biofilm-formation on diverse surfaces in shrimp larval tanks. This is of considerable importance as these materials are found in medical, farming and industrial environments and are constantly available for bacterial adherence, colonization and biofilm-formation.

In order to more closely simulate natural conditions, flow cell and bioreactor systems have been devised (Banning *et al.*, 2003; Bremer *et al.*, 2002; Hall-Stoodley and Lappin-Scott, 1998; and Wang *et al.*, 2003). Flow cell systems have been developed for the microscopic investigation of the biofilm formation of microorganisms under continuous flow conditions. Different flow cell models have been used for this purpose, however, flow cell systems generally consist of inoculated chambers or channels, which either contain removable components or are covered with glass slides to facilitate microscopy, and a pump to allow continuous flow of fresh medium through the inoculated chambers. Hence, flow cell systems allow detailed visual analysis of biofilm growth and the production of both quantitative and qualitative data.

A three-channel flow cell system has been used to determine the effect of an OMP on biofilm formation by the fish pathogen *V. anguillarum* (Wang *et al.*, 2003). A slightly modified device, involving the recirculation of a batch culture at a constant flow rate, has been described for the investigation of biofilm growth of rapidly growing mycobacteria (Hall-Stoodley and Lappin-Scott, 1998). However, only the use of a bioreactor has been described in an investigation regarding the effect of chlorine on mixed bacterial biofilms comprised of *Flavobacterium* spp. and *L. monocytogenes* (Bremer *et al.*, 2002). The use of flow cell systems for the investigation of *Flavobacterium* spp. could reveal valuable information about the adherence capabilities and therefore pathogenesis of certain species. The flow cell investigation of both single and mixed biofilms formed by pathogenic *Flavobacterium* spp. and other aquatic organisms may also reveal important information on the behaviour and interaction of pathogenic *Flavobacterium* spp. in their natural environment and could in part lead to possible explanations for recurrent *Flavobacterium* disease outbreaks.

Microorganisms inhabiting aqueous environments are well known for their biofilm-forming abilities (Bell, 2001; Donlan, 2002; Stickler, 1999; and Webb *et al.*, 2003). Initial attachment of such biofilm-forming cells is facilitated by expression of cell surface polymers, which alter cell surface properties (Rickard *et al.*, 2004). Cell surface structures such as flagella, pili, fimbriae, surface

proteins and capsule play an important role in biofilm initiation (Donlan, 2002, Stickler, 1999). Many of these structures are important components of bacterial motility and non-motile mutants have been shown to lack biofilm-forming ability, compared to wild type cells (Stickler, 1999). Certain properties which have been found to enhance bacterial attachment, including increased whole cell hydrophobicity and the ability to coaggregate and autoaggregate (Decostere *et al.*, 1999b; Rickard *et al.*, 2004, and Van der Mei *et al.*, 2003). High proportions of bacterial species, not detectable in the surrounding fluid phase, have been detected in biofilms formed at high shear rates (Rickard *et al.*, 2004). Additionally, a larger proportion of biofilm strains, compared to their planktonic counterparts, have been found able to coaggregate and autoaggregate (Rickard *et al.*, 2004). These cell surface properties, along with differences in shear rate, profoundly affect biofilm-associated microbial diversity and may play an important role in the potential of opportunistic pathogens to integrate into freshwater biofilms (Rickard *et al.*, 2004). As mentioned above many aquatic pathogens, such as *V. anguillarum*, *V. harveyi*, and *Y. ruckeri*, causing great losses in aquaculture farming, have been detected in biofilms present in aquaculture settings (Coquet *et al.*, 2002a; Karunasagar *et al.*, 1996; O'Sullivan *et al.*, 2002; Wang *et al.*, 2002; and Wang *et al.*, 2003).

The recurrence of disease and infection caused by pathogenic bacteria present in aquaculture systems has been ascribed, in part, to their biofilm-forming abilities (Coquet *et al.*, 2002a; Karunasagar *et al.*, 1996). *Flavobacterium* spp. are important pathogens in the aquaculture setting (Bernardet and Bowman, 2005) and have also been isolated from a number of biofilms present in industrial environments (Oppong *et al.*, 2003). Given that aquaculture surfaces are easily colonized, persisting *Flavobacterium* spp. inhabiting biofilms might serve as a source of infection or re-infection. In Chapter 3, *Flavobacterium* spp. isolates of aquaculture origin were found to display heterogeneity with respect to their molecular and chemotaxonomical profiles, which might be correlated with differences in the ability to form biofilms. The present study thus investigated the ability of various *Flavobacterium* spp.

isolates, isolated from diseased fish and biofilms within aquaculture tanks, to form biofilms using microtiter-plate adherence assays, as well as flow cell investigations.

4.2 MATERIALS AND METHODS

4.2.1 Microtiter adherence assays

Wells of 96 well microtiter-plates, U-shaped wells (Bibby Sterilin Ltd, UK), were inoculated with 100 μ l of overnight EAOB cultures (inoculum standardised to a 0.5 McFarland standard). Negative control wells contained broth only and positive control wells contained 100 μ l of the *Vibrio* spp. (inoculum standardised to a 0.5 McFarland standard). Microtiter-plates were incubated for 24 h at room temperature and 26°C, respectively, with gentle shaking. The procedure was performed in triplicate for each isolate. Following incubation, well contents were discarded and wells were washed three times with 250 μ l of sterile physiological saline. Plates were vigorously shaken to remove non-adherent bacteria, after which the biofilms were fixed with 200 μ l of 99% methanol. After 15 minutes, plates were emptied and left to dry. Plates were then stained with 200 μ l of 2% crystal violet and left for 5 minutes. Excess stain was removed with running tap water and plates were air-dried. Remaining bound dye was resolubilized with 160 μ l of 33% (v/v) glacial acetic acid (Stepanovic *et al.*, 2000). Optical densities of wells were measured at 595 nm before and after the addition of glacial acetic acid using a Bio-Rad Microplate Reader (Model 680, Bio-Rad Laboratories, USA).

For comparative analysis of results, the adherence capabilities of isolates were divided into four categories (Stepanovic *et al.*, 2000). Based on the OD readings of biofilm growth, isolates were classified into the following categories: non-adherent (0), weakly adherent (+), moderately adherent (++) or strongly adherent (+++). The cutoff OD (OD_c) was defined as three standard deviations

above the mean OD of the negative control. Therefore, classification of isolates was carried out as follows:

$OD \leq OD_c$	non-adherent
$OD_c < OD \leq 2 \times OD_c$	weakly adherent
$2 \times OD_c < OD \leq 4 \times OD_c$	moderately adherent
$4 \times OD_c < OD$	strongly adherent

The three test results obtained for each isolate were averaged (Stepanovic *et al.*, 2000).

4.2.2 Biofilm-forming ability in flow cell systems

Following phenotypic and molecular characterisation studies in Chapters 2 and 3, *Flavobacterium* spp. isolates YO10, YO19, YO51, YO60 and YO64 were selected for further study using the flow cell system. A *Vibrio* spp. isolate obtained from an aquaculture biofilm was used as a biofilm-positive control (Karunasagar *et al.*, 1996; O'Sullivan *et al.*, 2002; Wang *et al.*, 2002; and Wang *et al.*, 2003). In addition to using the *Vibrio* spp. isolate as a positive control, an *A. hydrophila* isolate obtained from diseased trout was used to examine mixed bacterial biofilm growth, in combination with isolates YO19 and YO51, respectively, using the flow cell system. Eight channel flow-cell systems were used to test the biofilm-forming abilities of various isolates (Wang *et al.*, 2003). The eight-channel perspex flow cell (channel size $30 \times 4,5 \times 3$ mm) covered with a glass coverslip (Lasec, RSA) and with silicone tubing (1×1.6 mm \times 3 mm \times 5 m tubing; The silicone tube, RSA), attached with clear silicone sealant, were presterilized with liquid bleach for 1 h. The silicone tubing was connected, with the use of plastic adapters (T-connectors, 1/16"; straight connectors, 1/16" \times 1/16"; Cole-Parmer Instrument Co., USA), to a reservoir containing 2 l of EAOB. A pump (Watson-Marlow, UK) was fitted on the silicone tubing, upstream of the flow cell. The flow cell was filled with EAOB and the flow rate was stabilized to 3 rpm for 1 h. Clamps were used to seal silicone tubes upstream of each channel.

Sterile syringes with needles were used to inoculate a volume of 1 ml pure overnight culture of each of isolates YO10, YO19, YO51, YO60 and YO64, the *Vibrio* spp. isolate and *A. hydrophila* isolate, respectively, in each channel, below the clamps. Flow cell systems were kept at room temperature throughout the experiment. Each flow cell channel was investigated microscopically using a Nikon Eclipse E400 (Nikon, Japan) after a 24 h and 48 h time period, respectively, to visualize bacterial attachment and biofilm growth.

Mixed pure culture inoculations, including combinations of isolate YO19 and isolate YO51 with each of the *Vibrio* spp. isolate and the *A. hydrophila* isolate, respectively, comprised of 0.5 ml overnight growth of each organism to form a total volume of 1 ml. Stagnant conditions were maintained for the first hour, before inoculated channels were exposed to flowing EAOB at a constant flow rate of 3 rpm and 6 rpm, respectively. Negative and positive controls were subjected to the same conditions and comprised of sterile channels, with no added culture, and *Vibrio* spp. isolate inoculated channels, respectively.

A second flow cell system, carrying water from rainbow trout aquaculture tanks, was used to investigate mixed biofilms formed by the diversity of bacteria present in the aquaculture tank water. A number of organisms, including *Flavobacterium* spp., had been identified following sampling of tank water on EAOA. Other organisms (heterotrophs/pathogens) present in the aquaculture system were identified with the API identification assays and included mainly *Vibrio* spp., *Aeromonas* spp. and *Pseudomonas* spp. Sterile reservoirs containing 2 l of tank water were linked to flow cells as described previously, and a constant flow rate of 3 rpm was maintained. Additionally, a flow cell experiment was carried out using a combination of isolate YO19 and untreated tank water and isolate YO19 and autoclaved tank water, respectively. Therefore, the ability of isolate YO19 to form mixed biofilm growth, with other bacterial species present in tank water, and in sterile tank water, representing a more natural nutrient poor environment, was investigated.

4.2.3 Capsule stain

In order to investigate the presence of a capsule, Anthony's Capsule Staining method (Harley and Prescott, 1996) was performed on overnight EAOB cultures of isolates YO10, YO19, YO51, YO60 and YO64. A *Klebsiella pneumoniae* strain grown in brain-heart infusion (BHI) broth (Biolab diagnostics, RSA) was used as a control.

4.3 RESULTS

4.3.1 Microtiter-plate adherence assays

The optical density results obtained in triplicate, after the addition of acetic acid, tended to be uniform for each strain tested in the microtiter-plate adherence assays (Table 4.1 and Figs. 4.1-4.2). The *Vibrio* spp., used as control, displayed strong adherence at both 26°C and room temperature. Majority of the isolates tested displayed identical levels of adherence at both 26°C and room temperature. However, slight differences were obtained in adherence categories for certain flavobacterial isolates at the different incubation temperatures. Both isolates YO52 and YO56 displayed moderate adherence at 26°C and strong adherence at room temperature. Additionally, isolate YO57 displayed weak adherence at 26°C and moderate adherence at room temperature. A strong degree of adherence was consistently displayed by the following isolates viz., YO19, YO20, YO21, YO26, YO34, YO35, YO38, YO59, YO63, and YO64. Moderate adherence was obtained only for isolates YO45 and YO66. However, the majority of isolates tested demonstrated weak adherence throughout the microtiter-plate adherence assays. None of the strains tested were categorized as non-adherent, which indicated that all flavobacterial isolates were able to adhere variably to the PVC surface of the microtiter-plates.

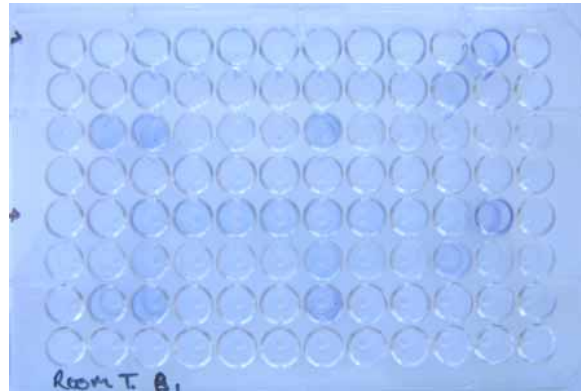


FIG. 4.1. Room temperature microtiter-plate adherence assay, where different shades of bound crystal violet display different levels of adherence, i.e., darker blue showing stronger adherence, by *Flavobacterium* spp. isolates prior to the addition of acetic acid.

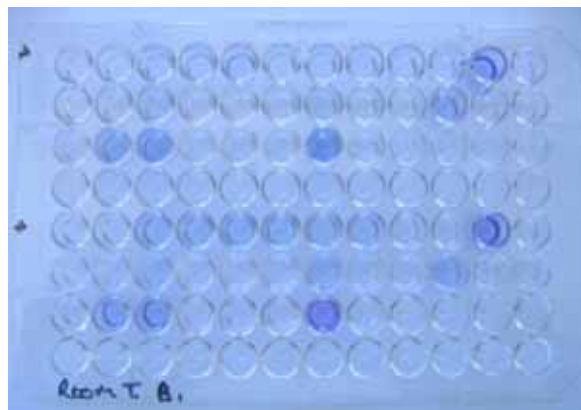


FIG. 4.2. Room temperature microtiter-plate adherence assay, where different shades of bound crystal violet display different levels of adherence, i.e., darker blue showing stronger adherence, by *Flavobacterium* spp. isolates following the addition of acetic acid.

TABLE 4.1. Microtiter-plate adherence assay results of *Flavobacterium* spp. isolates.

Isolate	Degree of adherence		WCP protein profile
	26°C	Room Temperature	
YO10	N/A*	N/A	N/A
YO11	N/A	N/A	N/A
YO12	W†	W	A1¶
YO15	W	W	A2
YO19	S‡	S	B**
YO20	S	S	B
YO21	S	S	B
YO26	S	S	B
YO34	S	S	B
YO35	S	S	B
YO38	S	S	B
YO45	M§	M	A1
YO46	N/A	N/A	A1
YO49	W	W	A1
YO50	W	W	A1
YO51	W	W	C††
YO52	M	S	B
YO53	W	W	A1
YO54	W	W	A1
YO55	W	W	B
YO56	M	S	B
YO57	W	M	A2
YO58	W	W	A2
YO59	S	S	B
YO60	W	W	A2
YO61	W	W	A2
YO62	W	W	A2
YO63	S	S	B
YO64	S	S	B
YO65	W	W	C
YO66	M	M	B
YO67	W	W	C

* Cultures not available for testing (N/A)

† Weakly adherent (W)

‡ Strongly adherent (S)

§ Moderately adherent (M)

¶ Whole cell protein profile A1

|| Whole cell protein profile A2

** Whole cell protein profile B

†† Whole cell protein profile C

4.3.2 Flow cell study

4.3.2.1 Biofilm-formation by single, pure-culture *Flavobacterium* isolates and controls

Microscopic analysis of monocultures of *Flavobacterium* spp. isolates YO10, YO19, YO51, and YO64 tested for biofilm formation, revealed biofilm structures on the glass slides within 48 h. However, thick biofilm growth in the

form of orange carpets was observed, without the use of microscopy, on perspex channel surfaces for all flavobacterial isolates tested within the first 24 h following incubation. As with the microtiter-plate adherence assays, the flow cell study revealed variation among the isolates with respect to biofilm formation.

For isolate YO10, biofilm production was only observed 48 h following inoculation. Bacterial association with the glass slide was observed within the first 24 h, which revealed gliding motility across the surface of the glass slide (Fig. 4.3).

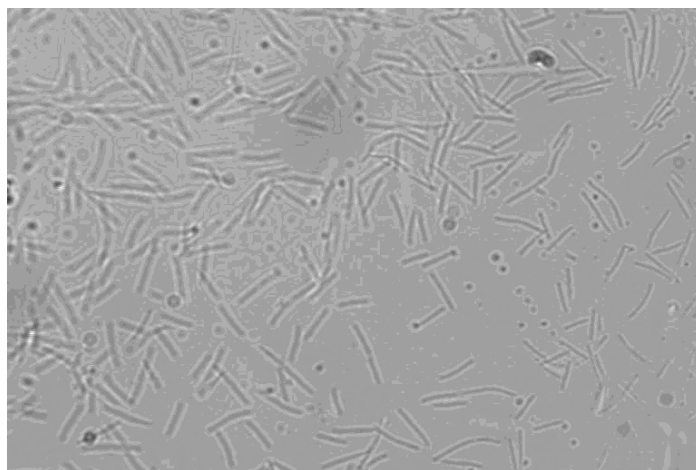


FIG. 4.3. Light microscope image depicting cells of isolate YO10 associated with the glass slide surface following 24 h flow cell incubation (1000 \times magnification).

After 48 h, isolate YO10 formed micro-colonies with filaments extending into the flowing medium. Micro-colonies were pillar-shaped and also extended into the flowing media (Fig. 4.4).

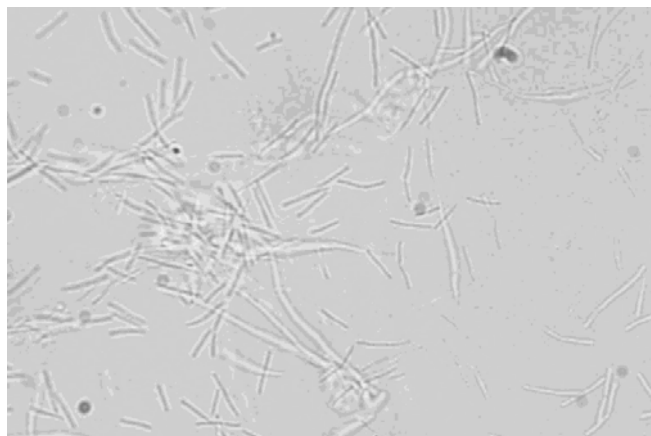


FIG. 4.4. Light microscope image of isolate YO10, where pillar-shaped micro-colonies with filaments extending into the flowing medium were observed in flow cell chambers following 48 h flow cell incubation (1000× magnification).

Of the four *Flavobacterium* spp. isolates tested by flow cell studies, isolate YO19 appeared to attach to the glass slide and form biofilms most rapidly. No gliding motility was observed during the initial stages of attachment to the glass slide surface. Mono-layered biofilms produced by isolate YO19 were observed within the first 24 h following inoculation (Fig. 4.5). Majority of isolate YO19 cells attached to the glass surface along their length, appearing stationary.

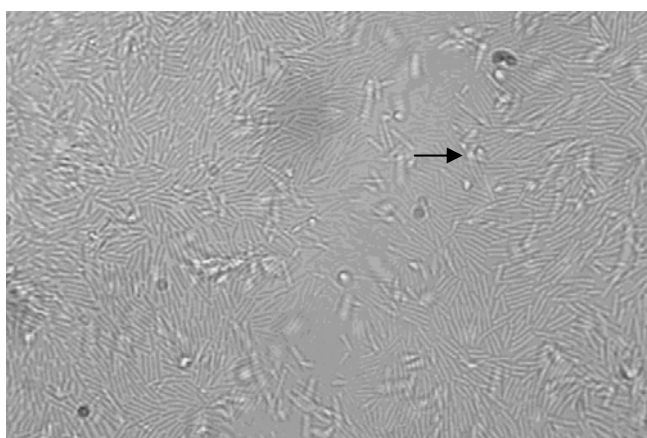


FIG. 4.5. Light microscope image depicting mono-layered biofilm formation by isolate YO19, including polar-attached cells (arrow), following 24 h flow cell incubation (1000× magnification).

Certain cells appeared to be polar attached to the glass surface and seemed to rotate in place (Fig. 4.6). The polar attachment and rotation was also observed for isolate YO51, albeit to a minor extent, and isolate YO64. After 48 h, thick layers of biofilm cells were observed, covering most of the glass slide surface.

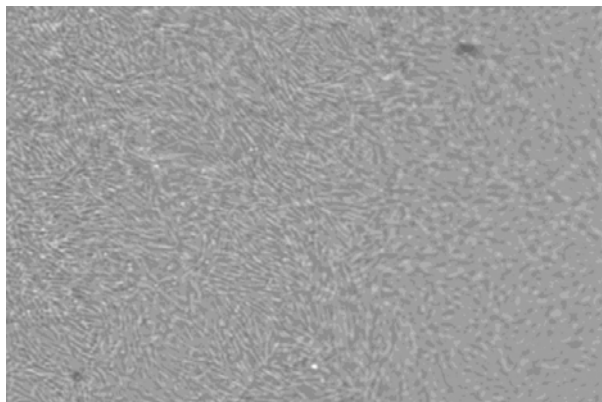


FIG. 4.6. Light microscope image depicting multi-layered biofilm formation by isolate YO19 following 48 h flow cell incubation (1000 \times magnification).

The surface of the biofilms in contact with the flowing media appeared irregular with pillar-shaped extensions protruding into the flowing media (Fig. 4.7). In certain areas, scattered, attached cells of isolate YO19 were still present on the glass slide surface after 48 h.

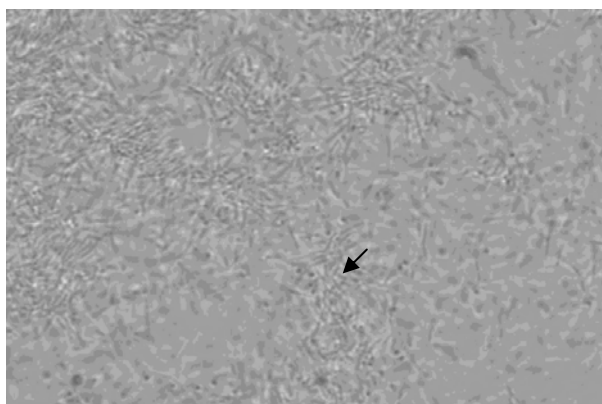


FIG. 4.7 Light microscope image depicting the irregular surface of a biofilm formed by isolate YO19 with pillar-like structures (arrow) protruding into the flowing medium following 48 h flow cell incubation (1000 \times magnification).

The production of micro-colonies, similar to those of isolate YO10, was observed within the first 24 h by increasing the flow rate to 6 rpm (Fig. 4.8).

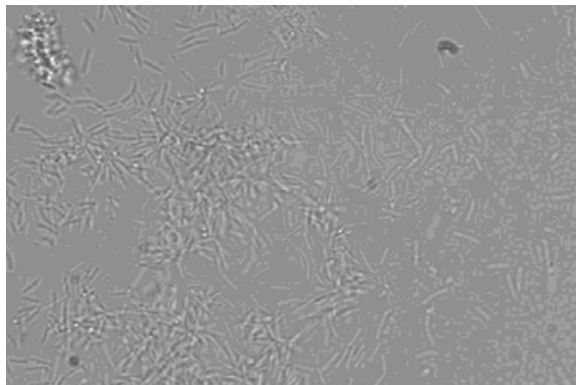


FIG. 4.8. Light microscope image depicting micro-colony formation by isolate YO19 at an increased flow rate of 6 rpm following 24 h flow cell incubation (1000 \times magnification).

Micro-colonies increased in size after 48 h, however, no layered biofilm formation by isolate YO19 was observed (Fig. 4.9). Long filaments were also present among the micro-colonies, usually extending into the flowing media. Due to its superior biofilm-forming ability, isolate YO19 was the only isolate subjected to variations in flow rate.

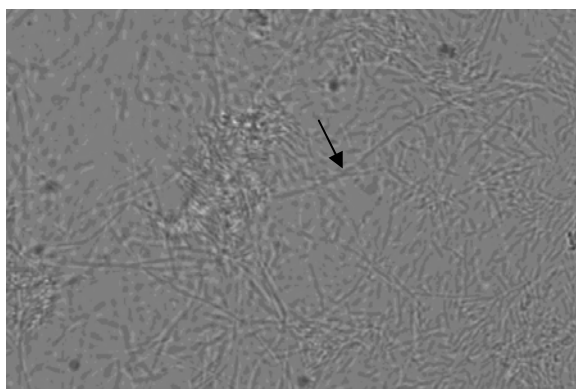


FIG. 4.9. Light microscope image depicting increased micro-colony formation by isolate YO19 at a flow rate of 6 rpm following 48 h flow cell incubation. Long filaments (arrow) extending into flowing media can be seen (1000 \times magnification).

The production of biofilms by isolate YO51 was only observed after 48 h. Cells of isolate YO51 associated with the glass surface along their length and revealed rapid gliding motility within 24 h following inoculation (Fig. 4.10).

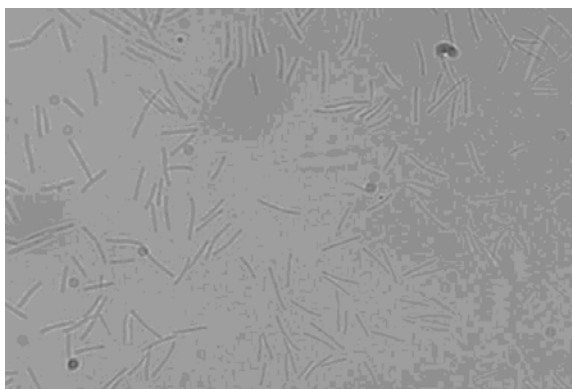


FIG. 4.10. Light microscope image depicting cells of isolate YO51 associated with the glass slide surface following 24 h flow cell incubation (1000× magnification).

Biofilm-formation by isolate YO51 appeared more prominent along the edges of the glass slide, where the perspex and silicone met with the glass slide, and thick, multi-layered biofilm production was observed (Fig. 4.11).

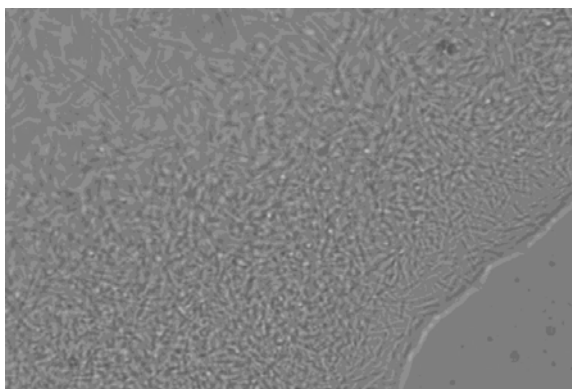


FIG. 4.11. Light microscope image depicting thick multi-layered biofilm growth by isolate YO51 at the glass-perspex interface following 48 h flow cell incubation (1000× magnification).

No biofilm formation was observed by isolate YO60 after 48 h following incubation. As with isolate YO51, cells of isolate YO60 associated with the glass surface displaying rapid gliding motility within 24 h following incubation (Fig. 4.12).

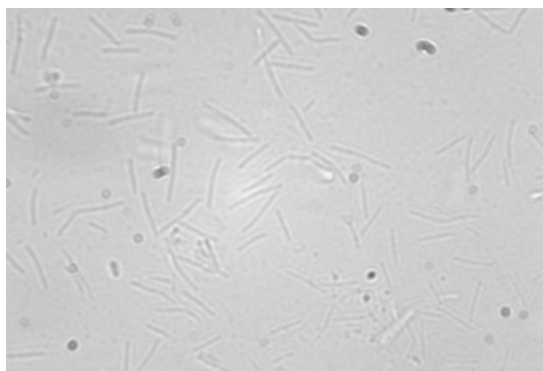


FIG. 4.12. Light microscope image depicting cells of isolate YO60 associated with the glass surface following 24 h flow cell incubation (1000× magnification).

The formation of a single micro-colony by isolate YO60 cells was observed after 24 h (Fig. 4.13). More cells associated with the glass surface after 48 h, however, the majority of these cells appeared stationary (Fig. 4.14). Only a small number of gliding cells were present among stationary attached cells. No further micro-colony formation was observed after 48 h.

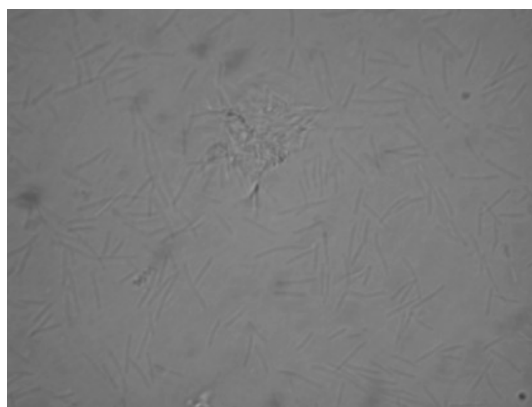


FIG. 4.13. Light microscope image depicting micro-colony formation by cells of isolate YO60 following 24 h flow cell incubation (1000× magnification).

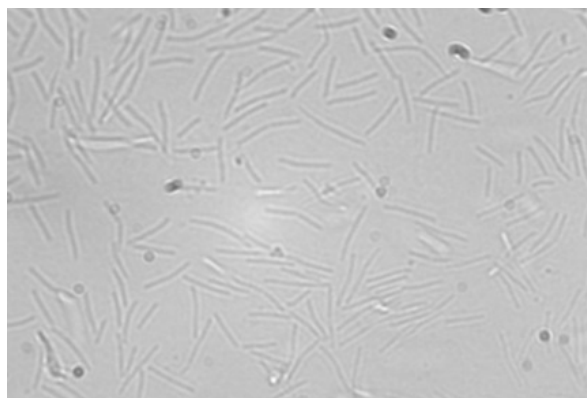


FIG. 4.14. Light microscope image depicting increased amounts of glass surface-associated cells of isolate YO60 following 48 h flow cell incubation (1000× magnification).

Cells of isolate YO64 displayed gliding motility and polar attachment to the glass slide surface within 24 h following inoculation (Fig. 4.15). Biofilm formation by isolate YO64 was observed after 48 h as mono- and multi-layered growth with abundant polar attached cells (Fig. 4.16). Certain areas of the glass slide surface, among mono-layered biofilm growth, remained colonized with scattered attached cells.

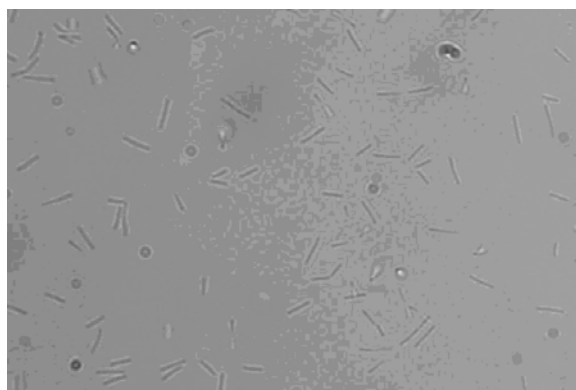


FIG. 4.15. Light microscope image depicting glass surface-associated cells of isolate YO64 with randomly spread polar-attached cells following 24 h flow cell incubation (1000× magnification).

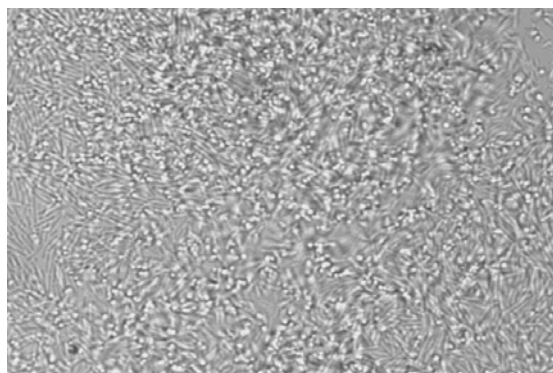


FIG. 4.16. Light microscope image depicting mixed mono- and multi-layered biofilm-formation by isolate YO64 with abundant polar-attached cells following 48 h flow cell incubation (1000× magnification).

Both the *A. hydrophila* and *Vibrio* spp. isolates attached to the glass slide and formed biofilm structures within the first 24 h following inoculation. The *A. hydrophila* and *Vibrio* spp. isolates displayed a far more rapid rate of biofilm formation in comparison to the *Flavobacterium* spp. Differences were observed with respect to the biofilm formation characteristics and architectures for both *A. hydrophila* and *Vibrio* spp. isolates.

The *A. hydrophila* isolate attached and formed mono-layered biofilms within the first 24 h following inoculation (Fig. 4.17). Micro-colonies along with polar attached cells and long filaments, protruding into the flowing media, were observed.

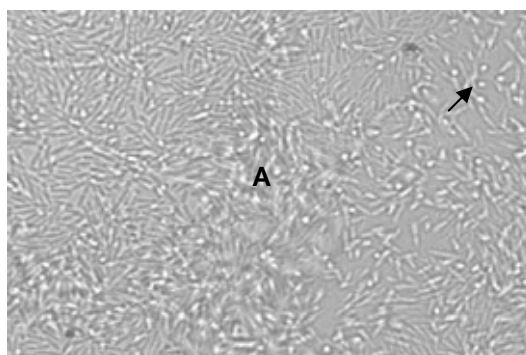


FIG. 4.17. Light microscope image depicting mono-layered biofilm-formation by the *A. hydrophila* isolate following 24 h flow cell incubation. Micro-colonies (A) and multiple polar-attached cells (arrow) can be seen protruding into the flowing medium (1000× magnification).

Thick, multi-layered *A. hydrophila* biofilms, uniformly covering the glass slide surface, were present after 48 h following inoculation (Fig. 4.18).

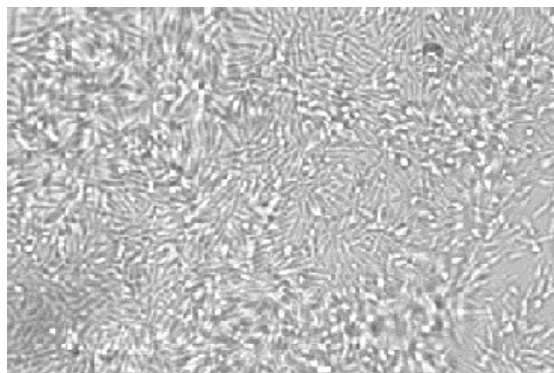


FIG. 4.18. Light microscope image depicting multi-layered biofilm-formation by the *A. hydrophila* isolate cells following 48 h flow cell incubation (1000× magnification).

Initial cell attachment of the *Vibrio* spp. isolate was observed after 24 h in the form of micro-colonies scattered randomly across the surface of the glass slide (Fig. 4.19).

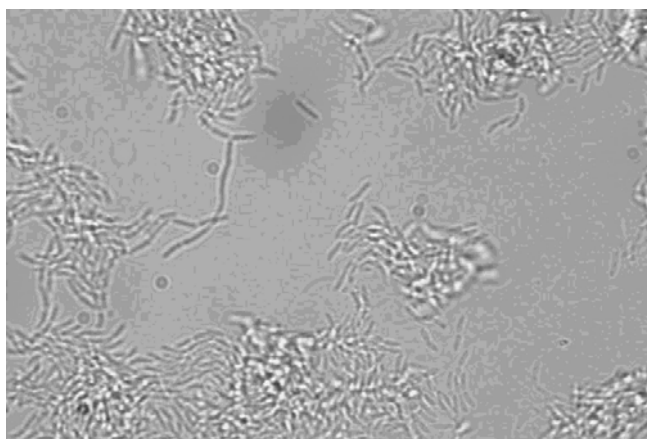


FIG. 4.19. Light microscope image depicting scattered micro-colonies formed by the *Vibrio* spp. isolate following 24 h flow cell incubation (1000× magnification).

A complex, multi-layered *Vibrio* spp. biofilm structure covering the entire surface of the glass slide was observed after 48 h (Fig. 4.20). Channels within the biofilm structure and pillar-shaped extensions protruding the flowing media on the outer surface of the biofilm were visible on examination.

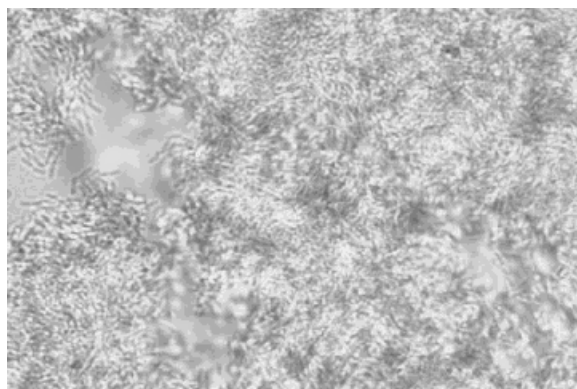


FIG. 4.20. Light microscope image depicting complex multi-layered biofilm-formation by the *Vibrio* spp. isolate following 48 h flow cell incubation (1000× magnification).

4.3.2.2 Biofilm-formation of mixed cultures of bacteria

When co-inoculated, both isolate YO19 and *A. hydrophila* cells appeared to randomly attach to the glass slide surface within the first 24 h following inoculation (Fig. 4.21).

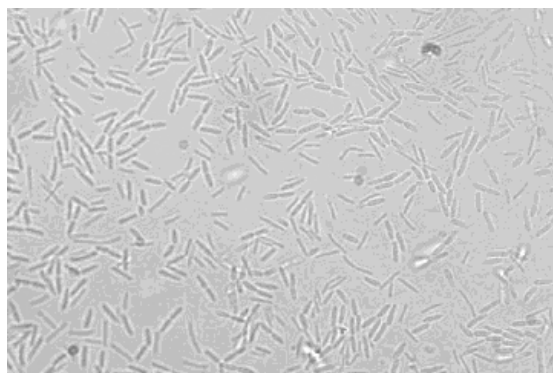


FIG. 4.21. Light microscope image depicting cells of isolate YO19 and *A. hydrophila* randomly attached to the glass surface following 24 h flow cell incubation (1000× magnification).

Biofilm formation by isolate YO19 was observed after 48 h in the form of scattered, multi-layered, pillar-shaped micro-colonies among mono-layers of *A. hydrophila* biofilm growth. *A. hydrophila* cells were spread among flavobacterial cells within the micro-colonies and also attached to the surface of the micro-colonies to form complex heterogeneous microcosms (Fig. 4.22).

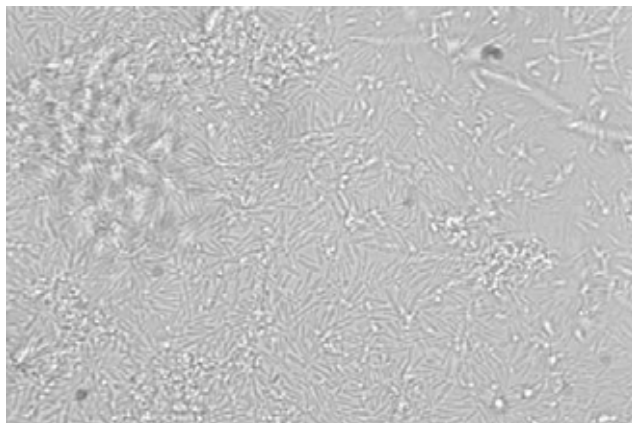


FIG. 4.22. Light microscope image depicting cells of isolate YO19 and *A. hydrophila* forming complex heterogeneous microcosms following 48 h flow cell incubation (1000× magnification).

When isolate YO51 was coupled with *A. hydrophila*, the *A. hydrophila* cells attached within 24 h (Fig. 4.23). As described for single-culture biofilms, isolate YO51 associated with the glass slide surface and revealed rapid gliding motility among attached *A. hydrophila* cells. Biofilm formation after 48 h by *A. hydrophila* cells was observed in the form of mono-layers partially covering the glass slide surface. Although gliding cells of isolate YO51 were present among the scattered, attached *A. hydrophila* cells on the glass slide, no attachment or biofilm formation was observed at any stage within 48 h following inoculation.

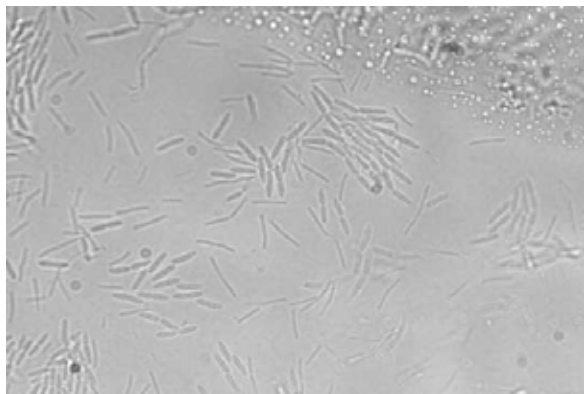


FIG. 4.23. Light microscope image depicting cells of isolate YO51 associated with the glass surface, randomly spread among attached *A. hydrophila* cells following 24 h flow cell incubation (1000× magnification).

A mixed culture study of *Flavobacterium* spp. isolate YO19 and the *Vibrio* spp. isolate, revealed distinct scattered micro-colonies formed by the *Vibrio* spp. isolate after 24 h, with randomly attached flavobacterial cells in between. Isolate YO19 formed multi-layered biofilms, resembling thick carpets after 48 h in certain areas (Fig. 4.24). Micro-colonies comprised of *Vibrio* spp. cells attached to the outer surface of isolate YO19 biofilm growth and extended towards the glass slide surface, forming a thick layer of complex growth containing channels and pillar-like structures protruding the flowing medium. Biofilms formed by both organisms seemed homogenous in appearance.

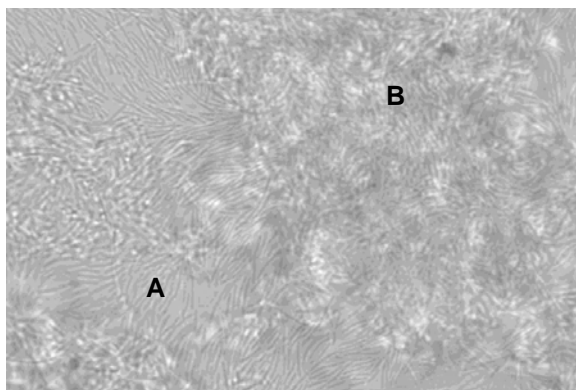


FIG. 4.24. Light microscope image depicting multi-layered biofilm-formation by isolate YO19 (A) and micro-colonies of the *Vibrio* spp. isolate (B) on the outer surface following 48 h flow cell incubation (1000× magnification).

When *Flavobacterium* spp. isolate YO51 and the *Vibrio* spp. isolate were combined, the *Vibrio* spp. cells displayed the same characteristic scattered micro-colonies, as described previously, after 24 h following inoculation (Fig. 4.19). Rapidly gliding cells of isolate YO51 were observed among the micro-colonies formed by the *Vibrio* spp. isolate. Similar complex biofilm structures were observed as in the mixed biofilm growth of isolate YO19 and *Vibrio* spp. isolate after 48 h (Fig. 4.24). The base layers were formed by thick, tightly-packed cells of isolate YO51 with micro-colonies of *Vibrio* spp. on the outer surface, extending to the surrounding glass slide surface to form complex structures containing channels and pillar-like structures (Fig. 4.25).

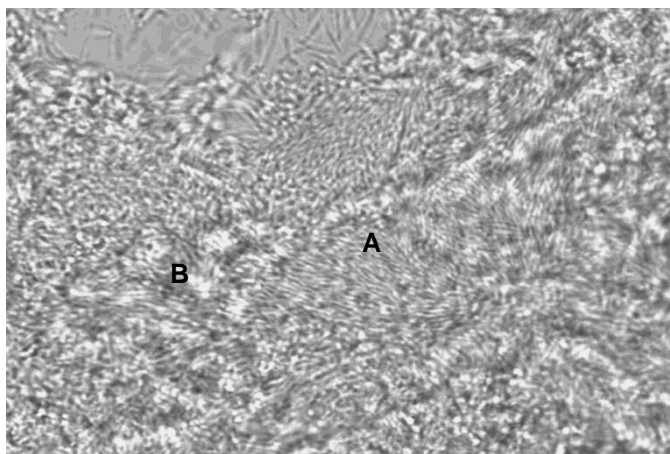


FIG. 4.25. Light microscope image depicting multi-layered biofilm-formation by isolate YO51 (A) and micro-colonies of the *Vibrio* spp. isolate (B) on the outer surface following 48 h flow cell incubation (1000× magnification).

4.3.2.3 Biofilm-formation of bacteria in flow cell systems inoculated with aquaculture tank water

Microscopy of tank water samples revealed the presence of diverse bacterial species and protists. Bacterial attachment to the glass slide occurred within the first 24 h following incubation. Biofilms, in the form of micro-colonies and large numbers of different cell types suspended in thick EPS layers forming complex, multispecies consortia, were also observed within the first 24 h and up to 48 h following incubation. Cell types associated with the glass slide varied dramatically in shape and a diversity of coccoid cells (Fig. 4.26), pleomorphic bacilli (Fig. 4.26), curved and spiral cells (Fig. 4.27) to long filamentous forms, were observed among micro-colonies and gliding across the glass slide surface (Fig. 4.28), was detected. Cells with a morphology resembling that of *Vibrio* spp. appeared suspended among other cell types in thick EPS layers (Fig. 4.29).

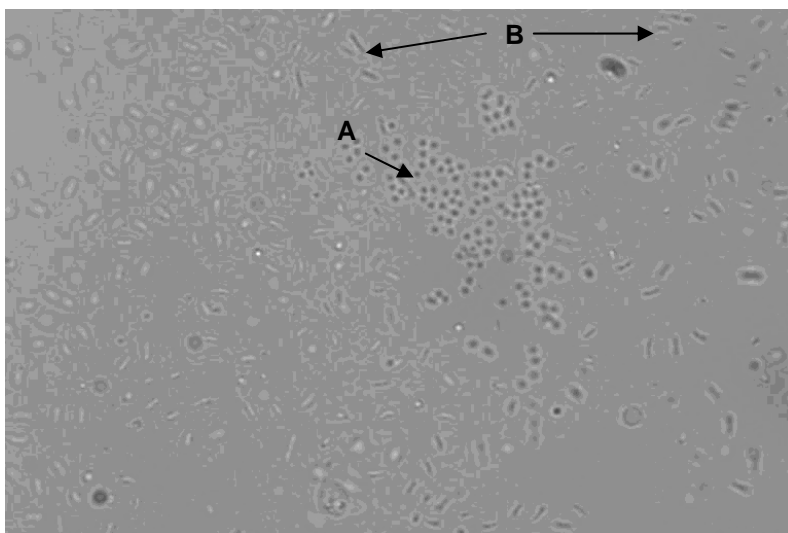


FIG. 4.26. Light microscope image of an aquaculture tank water sample depicting cocci (A) and pleomorphic bacilli (B) attached to the glass surface following 24 h flow cell incubation (1000× magnification).

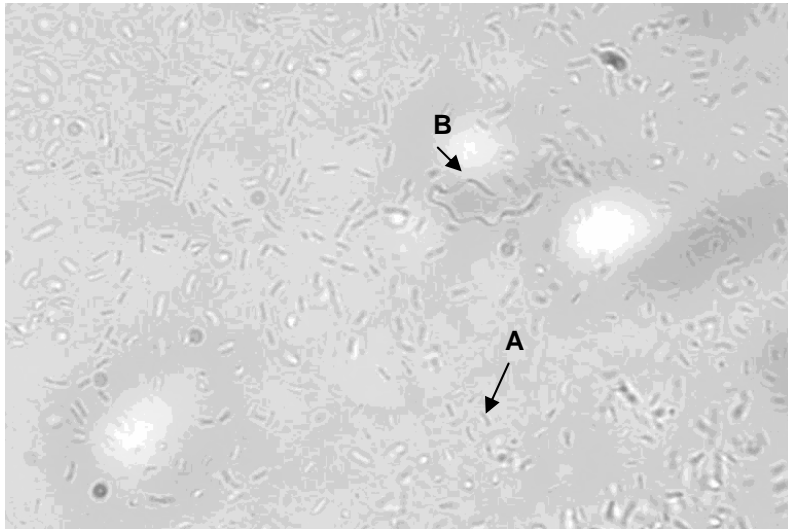


FIG. 4.27. Light microscope image of an aquaculture tank water sample depicting curved cells (A) and spiral cells (B) attached to the glass surface following 24 h flow cell incubation (1000× magnification).

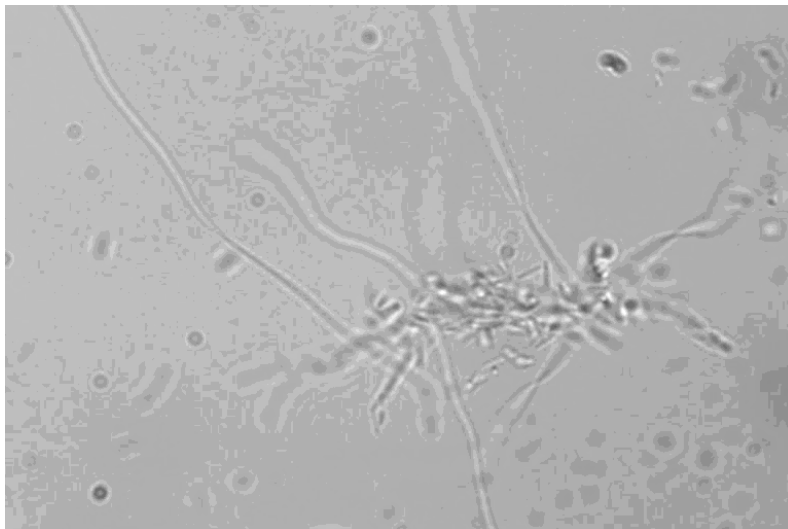


FIG. 4.28. Light microscope image of an aquaculture tank water sample depicting heterogeneous micro-colonies formed by diverse bacterial forms including long filamentous bacterial cells following 24 h flow cell incubation (1000× magnification).

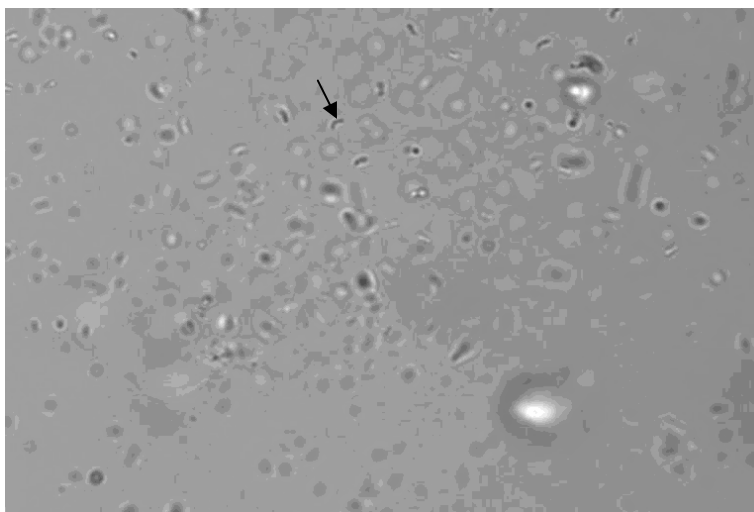


FIG. 4.29. Light microscope image of an aquaculture tank water sample depicting cells resembling that of *Vibrio* spp. (arrow) suspended among other cell types in a thick EPS layer following 48 h flow cell incubation (1000× magnification).

4.3.2.4 Biofilm-formation of isolate YO19 cells seeded into aquaculture tank water

In order to investigate the ability of flavobacterial isolate YO19 to be a competitive member of a mixed biofilm, water collected from a trout aquaculture system was seeded with isolate YO19. Without prior sterilization treatment, similar forms of bacterial growth were observed on the surface of the glass slide as observed in the native aquaculture tank water (Section 4.3.2.3, Figs. 4.26-4.29). Biofilm growth, in the form of micro-colonies and thick EPS layers, was detected after 24 h (Fig. 4.30). Scattered, attached bacterial cells were abundant among biofilm growth and micro-colonies. Many cell types associated with the glass slide surface were also observed including long slender bacilli resembling isolate YO19 (Fig. 4.30).

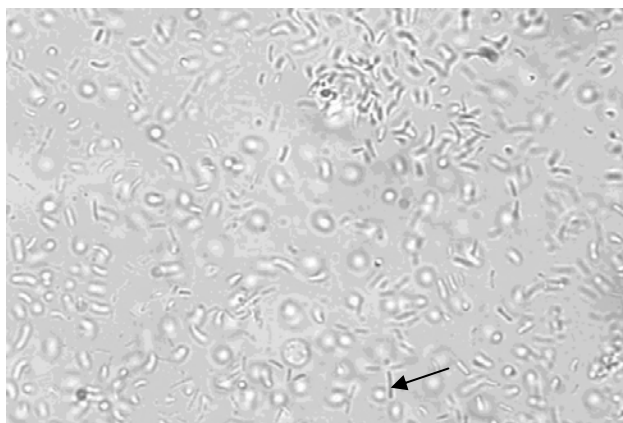


FIG. 4.30. Light microscope image of an aquaculture tank water sample seeded with isolate YO19 depicting biofilm formation by diverse bacterial cells suspended in a thick layer of EPS following 48 h of flow cell incubation. Long slender bacilli (arrow) resembling cells of isolate YO19 were visible among the multi-species consortia (1000 \times magnification).

A second sample of collected aquaculture tank water was autoclaved prior to inoculation with isolate YO19. In this instance, scattered bacterial attachment by isolate YO19 was observed after 24 h. Cell morphology varied from short, slender, pleomorphic bacilli to long, slender bacilli. Long, slender bacilli resembled isolate YO19 as observed in the single-culture flow cell study performed on isolate YO19 in EAOB (Fig. 4.31).

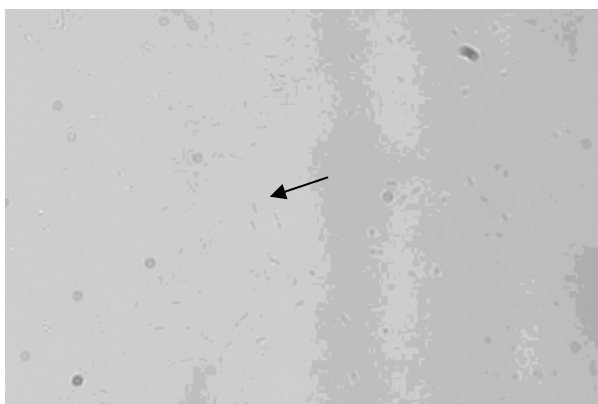


Fig. 4.31. Light microscope image of sterile aquaculture water inoculated with isolate YO19 depicting scattered attached pleomorphic cells (arrow) following 24 h of flow cell incubation (1000 \times magnification).

4.3.3 Capsule stain analysis

Capsule stains, performed on isolates YO10, YO19, YO51, YO60 and YO64, revealed the presence of capsule in all five isolates as well as the *K. pneumoniae* control. No noticeable difference in terms of capsule thickness was observed among flavobacterial isolates (Fig. 4.32).

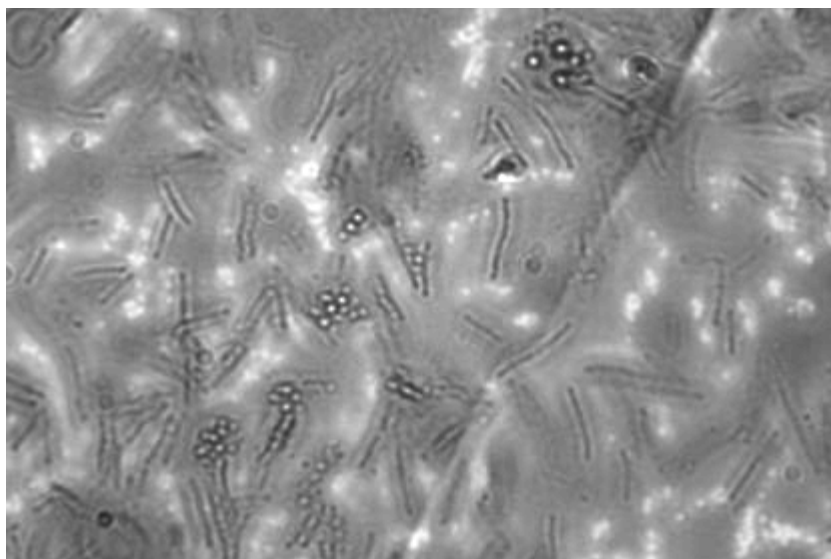


FIG. 4.32. Light microscope image depicting encapsulated cells of *Flavobacterium* spp. isolate YO10 (1000× magnification).

4.4 DISCUSSION

The survival of most aquatic pathogenic microorganisms, including *Flavobacterium* spp., outside of their hosts may be dependant on their biofilm-forming abilities (Coquet *et al.*, 2002a; and Wang *et al.*, 2002). Besides the pathogenic relationship certain *Flavobacterium* spp. share with fish, it is believed that many species of the genus *Flavobacterium* also form part of the natural microflora of fish (Inglis and Hendrie, 1993; and Bernardet and Bowman, 2005). Most of these *Flavobacterium* spp., pathogens and opportunistic pathogens

included, have been detected and identified in biofilm material collected from various aquatic environments (Bernardet and Bowman, 2005; Oppong *et al.*, 2003; and O'Sullivan *et al.*, 2002). Biofilm formation by *Flavobacterium* spp. may well be an adaptive advantage for survival in the aquatic environment. Aquaculture systems provide the ideal habitat especially for pathogenic bacteria to exist in biofilm structures, for not only is there a rich flow of nutrients and close proximity to the hosts, but a variety of surfaces amenable for bacterial colonization are also provided. The isolation and subsequent identification of three *Flavobacterium* spp. isolates YO65, YO66 and YO67, from an aquaculture tank biofilm (Chapter 2; Section 2.2; Table 2.1), is indicative of the presence of *Flavobacterium* spp. in biofilms as part of natural aquaculture systems.

The microtiter-plate adherence assay results showed positive adherence by all *Flavobacterium* spp. isolates tested at both room temperature and 26°C to the PVC surface, although the majority (49%) appeared to be weakly adherent (Table 4.1). Only 10% and 41% of the isolates were moderately or strongly adherent, respectively. Strong adherence, among other isolates, was observed for isolates YO19 and YO64. Isolates YO52, YO56 and YO57 displayed stronger adherence at room temperature compared to that observed at 26°C, indicating a lower optimal growth temperature for these isolates (Table 4.1). Isolates YO56 and YO57 had shown no and very weak growth, respectively, at 30°C straight after isolation, however, repeated temperature tests showed the adaptive nature of these *Flavobacterium* spp. isolates (Chapter 2, Section 2.3). It is, however, not unlikely that incubation at 26°C may have affected their adherence ability. The initial use of a microtiter-plate adherence assay described by O'Toole *et al.* (1999) generated results with poor reproducibility, prior to the use of the modified assay (Stepanovic *et al.*, 2000). The choice of microtiter-plate adherence assays is critical and must be assessed for different organisms. Data obtained in triplicate was uniform for each bacterial isolate tested in the present study using the modified microtiter-plate adherence assay described by Stepanovic *et al.* (2000), indicating consistency and accuracy of this technique.

Isolates YO10, YO19, YO51, and YO64, displayed a biofilm phenotype by both microtiter-plate adherence assays as well as using flow cell systems. However, microscopic analysis of the flow cells revealed important qualitative characteristics and differences displayed by these isolates. Interestingly, isolate YO51 only formed biofilms at the glass-perspex/silicone interface. This could be due to weak association with the glass surface by isolate YO51 or reduced flow along the edges of channels, enhancing the growth of isolate YO51 in these areas. Biofilm formation on the perspex walls of the flow cell channels, which seemed superior to biofilm growth on the glass slide surface, was also noted for all four strains tested. The superior bacterial association with the perspex surface could be attributed to difference in texture, such as roughness, and difference in charge or surface hydrophobicity, which enhanced bacterial attachment (Bell, 2001; Coquet *et al.*, 2002a; Donlan, 2002; Rickard *et al.*, 2004; Stickler, 1999; Van der Mei *et al.*, 2003; and Webb *et al.*, 2003). The *Flavobacterium* spp. isolates used in this study were found to be very hydrophilic (Basson, 2005), yet they showed high affinity to hydrophobic substances, such as perspex. This is in accordance with Coquet *et al.* (2002a) who reported *Y. ruckeri* cells to be hydrophilic, yet which showed affinity to both hydrophilic and hydrophobic surfaces.

Biofilms formed by each of the five *Flavobacterium* spp. isolates differed in terms of structure and morphology, from small micro-colonies formed by isolate YO10 (Fig. 4.4) to even, multi-layered biofilms formed by isolates YO19 and YO51 (Figs. 4.6 and 4.11) in the same time period. Surface attachment by isolate YO64 after 24 h revealed remarkable numbers of polar attached cells, rotating in place. Polar attached cells were also noted in the biofilm structures of isolates YO19 and YO51 at a much smaller scale. Increasing the flow rate for isolate YO19, resulted in altered morphology of biofilm structures compared to biofilms obtained at lower flow rates, however, this did not alter the rate of biofilm formation (Figs. 4.8-4.9). Rickard *et al.* (2004) found the flow rate to affect biofilm formation and biofilm structures of diverse freshwater bacteria.

Isolates YO19, YO51 and YO64, which showed superior biofilm formation compared to the other isolates, all exhibited the smooth colony morphology type and no gliding motility with the motility assays used in Chapter 2 (Section 2.3, Table 2.2). Isolates YO19 and YO64, also showed weak proteolytic activity for casein. However, during microscopic analysis of isolate YO51 and YO64, respectively, rapid gliding motility was observed. This was similar to isolate YO60, although, this isolate exhibited the hazy colony morphology type and weak gliding on agar. Qualitative microscopic analysis for determining the gliding ability of these organisms may be superior compared to the plate assays. A possible correlation between colony morphology type or gliding ability on agar and degree of adherence could not be established. However, a clear correlation was established between colony morphology type and the spreading nature on agar surface. These findings showed biofilm formation could be species or strain dependent and *Flavobacterium* spp. readily adapt to changing factors in their environment.

According to the 16S rRNA gene sequence data, *Flavobacterium* spp. isolates displaying a stronger degree of biofilm formation, i.e. isolates YO19 and YO64, shared 99% sequence similarity. The degree of adherence, or at least strong adherence in this case, may be strain-related. A very good correlation was established between WCP profile B and strong adherence, as all the *Flavobacterium* spp. isolates exhibiting WCP profile B showed strong biofilm formation in both the microtiter-plate adherence assays and flow cell studies (Table 4.1). Isolate YO66 was the only isolate exhibiting WCP profile B which displayed moderate adherence in the microtiter-plate adherence assays. A number of protein bands were unique to WCP profile B (Chapter 3, Section 3.3, Table 3.5). Some of these proteins may represent cell surface proteins, including glycoproteins, which facilitate bacterial attachment (Decostere *et al.*, 1999a; and Merle *et al.*, 2003). However, cell surface glycoproteins are also known to aid in virulence (Decostere *et al.*, 1999a; and Sara and Sleytr, 2000) and further investigation is needed to establish possible correlations between biofilm formation, WCP profiles and virulence of the *Flavobacterium* spp. isolates. It was

not possible to correlate specific OMP, RAPD and/or REP profiles with the biofilm phenotypes.

Very weak or no biofilm formation by flavobacterial isolates YO19 and YO51 was observed in mixed-culture flow cell experiments involving *A. hydrophila*. The rapid and even spread of *Aeromonas* cells across the surface of the glass slide, as seen in the pure culture flow cell study (Fig. 4.17), may have prevented the initial attachment and consequent biofilm formation by isolates YO19 or YO51. The absence and delay in biofilm formation by isolates YO51 and YO19, respectively, may also have been due to the release of inhibitory molecules by the *A. hydrophila* strain, for *Aeromonas* spp. are known for the production of bacteriocin-like substances (Bondi *et al.*, 2000; Lategan and Gibson, 2003; and Messi *et al.*, 2003). However, biofilm formation by isolate YO19, observed after 48 h, involved small complex heterogeneous micro-colonies consisting of both flavobacterial and *A. hydrophila* cells. In contrast, biofilm formation by flavobacterial isolates YO19 and YO51 appeared enhanced in mixed-culture flow cell experiments involving the *Vibrio* spp. isolate. Initial formation of scattered micro-colonies by the *Vibrio* spp. isolate may have facilitated attachment of flavobacterial isolates to the glass slide surface before the surface was sufficiently covered by *Vibrio* spp. cells. The different interactions in the mixed culture biofilms might involve differences in bacterial coaggregation. Certain cell surface characteristics, such as cell surface polymers, play an integral role in cell-to-cell adhesion (Kinder and Holt, 1994; and Shen *et al.*, 2005). Inter- and intra-species aggregation interactions were found to result in dental plaque, which consist of layered biofilms (Kinder and Holt, 1994; and Shen *et al.*, 2005). The coaggregation process is highly specific, involving the recognition of complementary lectin-carbohydrate molecules on the cell surface of aggregating cells (Kinder and Holt, 1994; and Shen *et al.*, 2005). In a coaggregation study, very weak coaggregation was found between isolate YO19 and various *Aeromonas* spp., including *A. hydrophila*, *A. salmonicida* and *A. sobria*, respectively (Basson, 2005). Variations with respect to lectin or carbohydrate moieties might explain the strong interaction between the *Vibrio*

spp. isolate and flavobacterial isolates and lack of interaction displayed by the *A. hydrophila* isolate and flavobacterial isolates, respectively.

No biofilm formation was observed by cells specifically resembling isolate YO19 in the aquaculture tank water seeded with this isolate (Fig. 4.30). Subculturing of bacterial isolates in the laboratory has been found to affect their biofilm-forming ability, leading to a loss in biofilm formation (Costerton, 2004). However, this was not the case for isolate YO19. Slow growth by isolate YO19 and competition with indigenous aquaculture microflora may have significantly affected biofilm formation by isolate YO19. The use of autoclaved tank water did not appear to affect attachment of isolate YO19 in a 24 h time period (Fig. 4.31). However, attached cells appeared deformed and smaller in size and lacked the ability to form biofilms after 48 h. Overall, the tank water did not induce biofilm formation by isolate YO19, although cell attachment was observed within the normal 24 h time period. Autoclaved tank water would be nutritionally poor compared to the EAOB and this might affect the overall growth rate and subsequent biofilm formation by isolate YO19.

Biofilm formation has long been associated with virulence in pathogenic bacteria (Bell, 2001; Donlan, 2002; Costerton, 2004; Stickler, 1999; and Webb *et al.*, 2003). The presence of many outer membrane structures, such as pili, fimbriae, outer membrane proteins, polysaccharides and other polymers, facilitating virulence also play an important role in biofilm formation (Bell, 2001; Donlan, 2002; Webb *et al.*, 2003; Stickler, 1999; Wang *et al.*, 2002; and Wang *et al.*, 2003). Capsule components have been shown to form part of the virulence mechanism of certain pathogenic *Flavobacterium* spp. (Decostere *et al.*, 1999a; and Decostere *et al.*, 1999b) and also play an important role in the adhesion and biofilm formation of certain bacterial species (Bell, 2001; Donlan, 2002; Stickler, 1999; and Webb *et al.*, 2003). According to Decostere *et al.* (1999a), the ability of *F. columnare* to adhere to gill tissue depended on a lectin-like carbohydrate-binding substance present in capsules found on the cell surface of the *F. columnare* strains tested. Transmission electron microscopy revealed the presence of capsule in both high and low virulent *F. columnare* strains

(Decostere *et al.*, 1999a). The high virulence strain revealed a thick capsule with a regular, dense appearance, whereas the capsule of a less virulent strain was much thinner and less dense (Decostere *et al.*, 1999a). The sticky nature of colonies formed by the *Flavobacterium* spp. isolates on EAOA may also be attributed to production of capsule and other extracellular polymers. However, in the present study no correlation could be established between the presence of capsule and the biofilm phenotype displayed by isolates YO10, YO19, YO51, YO60 and YO64, as capsules of similar thickness were produced by all five isolates. Congo red binds to an extracellular galactosamine glycan present in the extracellular slime produced by *F. columnare* (Bernardet and Bowman, 2005). The production of slime may also be linked to the synthesis of capsule components. All the *Flavobacterium* spp. isolates tested positive for the uptake of the Congo red dye in this study (Chapter 2, Table 2.2).

Although the virulence of pathogenic *Flavobacterium* spp. has been investigated, the role of biofilm formation as a virulence factor has not been examined at present (Decostere *et al.*, 1998; Decostere *et al.*, 1999a; Ekman, 2003; Kondo *et al.*, 2002; Michel *et al.*, 2003; and Thomas-Jinu and Goodwin, 2004). Consequently, no correlation has been established between virulence and the ability of *Flavobacterium* spp. isolated from diseased fish, to form biofilms. In order to identify possible correlations between virulence, biofilm-forming capabilities, and/or molecular and chemotaxonomical profiles, infection studies were carried out using selected isolates in a tilapia aquaculture system.

CHAPTER FIVE

EXPERIMENTAL INFECTIONS WITH *Flavobacterium* spp. ISOLATES IN A TILAPIA AQUACULTURE SYSTEM

4.1.1 INTRODUCTION

Aquaculture is based on the production of fish for both human consumption and sport fishing purposes. Commercial aquaculture suffers tremendous loss in fish worldwide, as a result of bacterial infections in both juvenile and mature fish. The economic impact of fish infections in large-scale aquaculture has led to an increasing interest in the bacterial pathogens responsible for disease.

Bacterial species frequently associated with infections in fish include *A. hydrophila*, *F. columnare*, *F. psychrophilum*, *V. anguillarum*, and *Y. ruckeri* (Holt *et al.*, 1993; Coquet *et al.*, 2002a; Wang *et al.*, 2002; Wang *et al.*, 2003; and Wakabayashi, 1993). Dalsgaard and Madsen (2000) monitored bacterial fish pathogens over a 2-year time period in five rainbow trout farms situated in Denmark. Although infections recorded were caused by *A. salmonicida*, *F. psychrophilum* and *Y. ruckeri*, respectively, infections caused by *F. psychrophilum* occurred most frequently (Dalsgaard and Madsen, 2000). Even though economic losses associated with fish mortality due to *F. psychrophilum* are higher compared to those caused by *F. columnare*, the latter remains one of the most important agents of bacterial disease in channel catfish and a variety of tropical ornamental fish species (Bernardet and Bowman, 2005; Decostere *et al.*, 1998; Decostere *et al.*, 1999b; and Michel *et al.*, 2002).

Fish in river systems may be infected by pathogenic *Flavobacterium* spp., however, farmed fish are particularly exposed to these pathogens due to high stocking densities and handling related stress (Bernardet and Bowman, 2005; and Dror *et al.*, 2005). Additionally, several host-related factors, such as skin or gill damage, close proximity and feed or oxygen deprivation, may influence the impact of flavobacterial disease in farmed fish populations

(Bernardet and Bowman, 2005). Recurrence of flavobacterial disease may be attributed to fish having survived an outbreak or resident fish species less susceptible, which may act as carriers or reservoirs of pathogenic *Flavobacterium* spp., and/or flavobacterial biofilm formation in the aquaculture environment (Bernardet and Bowman, 2005; and Coquet *et al.*, 2002a; Coquet *et al.*, 2002b; and Leonard *et al.*, 2000).

Columnaris disease is more prominent in summer when the optimum temperature range for infection, 20-30°C, is reached (Bernardet and Bowman, 2005). However, highly virulent *F. columnare* strains can cause disease at lower temperatures (Bernardet and Bowman, 2005). *F. psychrophilum*, on the other hand, only cause disease at low water temperature, usually below 10°C, which seems species-specific and infection rapidly becomes septicemic (Bernardet and Bowman, 2005). Other favobacterial infections, such as bacterial gill disease, occur at a wide range of temperatures and seem non-temperature dependant (Turnbull, 1993).

The symptoms displayed by infected fish can be linked to several virulence factors of the *Flavobacterium* spp. involved (Bernardet and Bowman, 2005). Pathogenicity of *F. columnare* and *F. psychrophilum* have been linked to the production of chondroitin AC lyase, which may be responsible for the extensive necrotic lesions observed in both columnaris disease and bacterial cold water disease (Bernardet and Bowman, 2005; and Stringer-Roth *et al.*, 2002). The production of extracellular proteases, including the metalloproteases Fpp1 and Fpp2 of *F. psychrophilum*, and hemagglutinating activities may also contribute to necrotic lesions and gill necrosis observed in flavobacterial fish infections (Bernardet and Bowman, 2005; Decostere *et al.*, 1999b; Holt *et al.* 1993; Lorenzen *et al.*, 1997; Secades *et al.*, 2001; and Secades *et al.*, 2003).

Adherence has also been demonstrated to play an integral role in bacterial attachment to gill tissue and may contribute to invasion of host tissue and virulence of *Flavobacterium* spp. (Bernardet and Bowman, 2005; Decostere *et al.*, 1999a; and Decostere *et al.*, 1999b). Cell surface components may also contribute to the virulence of certain *Flavobacterium* spp. Various other enzymatic activities, including the degradation of casein,

chitin, elastin, collagen and gelatin may contribute to the pathogenicity and virulence of fish pathogenic *Flavobacterium* spp. (Bernardet and Bowman, 2005; Holt *et al.*, 1993; and Turnbull, 1993)

Differences in virulence among pathogenic *Flavobacterium* spp. isolates are well documented (Decostere *et al.*, 1999a; Decostere *et al.*, 1998; Decostere *et al.*, 1999b; Madsen and Dalsgaard, 2000; Michel *et al.*, 2002; Stringer-Roth *et al.*, 2002; and Thomas-Jinu and Goodwin, 2004). Thomas-Jinu and Goodwin (2004) investigated suitable virulence markers in 17 *F. columnare* isolates obtained from six different fish species. Following the molecular characterisation of the *F. columnare* isolates, virulence of the isolates was determined by experimental bath infections with channel catfish in order to establish a possible correlation between virulence level and DNA markers (Thomas-Jinu and Goodwin, 2004). DNA markers used were not able to predict virulence, however, evidence of fish species-specific virulence was found (Thomas-Jinu and Goodwin, 2004). Additionally, virulence was found to be a strain-specific characteristic, indicative of variations in a small number of genes (Thomas-Jinu and Goodwin, 2004). Stringer-Roth *et al.* (2002) could not develop highly virulent *F. columnare* strains from low virulent strains by passage through salmonid fish and they concluded that the source of differences in virulence may be chromosomally-encoded.

A number of different infection study models have been used to investigate virulence levels of *Flavobacterium* spp. isolates. The use of experimental injection methods and bath infection studies have shown variable results, usually showing earlier onset of symptoms and higher mortality in the case of injection (Figueiredo *et al.*, 2005; and Madetoja *et al.*, 2003). Experimental infection studies using injection of pathogens are not optimal since certain components of innate immunity are overruled. Experimental bath infections simulate a more natural way of infection, however, no standard reliable experimental infection procedure, permitting good reproducibility of results, has been established to investigate virulence of *Flavobacterium* spp.

The present study made use of experimental bath infections, simulating more natural conditions, using Mozambique tilapia (*Oreochromis mossambicus*) as a model for temperate water fish infections, as the

Flavobacterium spp. isolates used in this study were all isolated from temperate environments (Chapter 2). Selected *Flavobacterium* spp. isolates characterized by molecular typing (Chapter 3), and biofilm formation (Chapter 4) were tested for differences in virulence, in order to establish possible correlations between molecular types, biofilm-forming capability and the virulence of *Flavobacterium* spp. isolates.

5.2 MATERIALS AND METHODS

5.2.1 Experimental Infections

Isolates YO12, YO19, YO51, YO60 and YO64 were experimentally tested for virulence against Mozambique tilapia (*Oreochromis mossambicus*). The tilapia fish were used as a model of temperate water farmed fish, simulating the environment isolates were obtained from (Chapter 2) and *Flavobacterium* spp. are known to infect tilapia (Figueiredo *et al.*, 2005). Bath immersion assays were carried out on tilapia. All assays were conducted in PVC aquaria containing 60 l aerated batch water. The final bacterial cell concentration of each respective isolate was 1×10^6 CFU/tank. *Flavobacterium* spp. isolates were grown overnight in 500 ml EAOB at 26°C. Bacteria were harvested by centrifugation at 8 000 rpm for 20 min and washed twice in sterile dH₂O. The required concentrations of bacterial suspensions were adjusted by measurement of the optical density at 525 nm (Michel *et al.*, 2002). Real infectious doses were assessed by plate enumeration of dilution series of each isolate prior to the infection study. Fish were divided into 6 groups of 30 fish (average size 10 cm). One group served as control and the rest were exposed to bath immersions of each respective isolate. Three different experiments were carried out on 3 different occasions. In the first experiment, fish were exposed to severe handling stress (double handling stress). In the second experiment, fish were exposed to mild handling stress (single handling stress) and the third experiment comprised of unstressed fish. In the first two experiments, bacterial suspensions were added immediately after fish were placed in aquaria. Fish used in the third

experiment were left to acclimatise for 72 h prior to addition of bacterial suspensions. Fish were examined daily for clinical signs of infection during a 10 day period. Water temperature was recorded every 24 h for 10 days. Dead fish were processed for re-isolation of *Flavobacterium* spp. isolates and samples were taken from gills, fins, liver and the spleen and streaked out onto EAOA. Water samples taken after 10 days from each tank, respectively, were also spread onto EAOA. All three experiments were carried out double blind-folded. Prior to the infection study, 10 random tilapia were sampled for the presence of *Flavobacterium* spp.

5.2.2 RAPD Fingerprinting

In order to confirm the identity of *Flavobacterium* spp. isolates YO12, YO19, YO51, YO60 and YO64, respectively, RAPD-PCR was performed on all the re-isolated bacteria morphologically resembling *Flavobacterium* spp. as well as stock cultures of isolates used in the infection study. RAPD-PCR and analysis of resulting RAPD fingerprints was carried out as described previously in Chapter 3, Section 3.2.5. RAPD fingerprinting was chosen due to its superior discriminatory index (91.93%) and the higher number in bands present in profiles compared to REP fingerprinting.

5.3 RESULTS

5.3.1 Experimental Infection and Virulence Analysis

Results obtained during all three infection experiments are shown in Table 5.1. The highest mortality rate and most severe symptoms were observed in highly stressed fish (experiment 1). Symptoms were observed after 72 h in the form of caudal fin rot (Fig 5.1), which dramatically increased in the following 48 h time period. Skin ulcers were also observed after 96 h (Fig. 5.2).

TABLE 5.1. Experimental infection of Mozambique tilapia (*Oreochromis mossambicus*) with *Flavobacterium* spp. isolates.

TABLE 3.1. Experimental infection of Mozambique tilapia (<i>Oreochromis mossambicus</i>) with <i>Vibrio</i> spp. isolates.												
Infection experiment number	Isolate	Tank number	Mortality* number and symptoms	(A total number of 30 fish were used in each tank)								
				Day 1 (24 h)	Day 2 (48 h)	Day 3 (72 h)	Day 4 (96 h)	Day 5 (144 h)	Day 6 (192 h)	Day 7 (240 h)	Day 8 (288 h)	Day 9 (336 h)
1	YO12	6	14*	1		Caudal fin rot	2	Caudal fin rot /skin ulcers	4	Severe caudal fin rot /skin ulcers	6	Severe caudal fin rot /skin ulcers
	YO19	1	10	3	1	Caudal fin rot	1	Caudal fin rot /skin ulcers	1	Severe caudal fin rot /skin ulcers	10	Severe caudal fin rot /skin ulcers
	YO51	5	13	1	0	Caudal fin rot	1	Caudal fin rot /skin ulcers	2	Severe caudal fin rot /skin ulcers	4	Severe caudal fin rot /skin ulcers
	YO60	4	18	3	2	Caudal fin rot	2	Caudal fin rot /skin ulcers	0	Severe caudal fin rot /skin ulcers	4	Severe caudal fin rot /skin ulcers
	YO64	2	13	0	2	Caudal fin rot	2	Caudal fin rot /skin ulcers	2	Severe caudal fin rot /skin ulcers	7	Severe caudal fin rot /skin ulcers
	Control	3	9	1	0	Caudal fin rot	1	Caudal fin rot /skin ulcers	3	Caudal fin rot /skin ulcers	7	Caudal fin rot /skin ulcers
2	YO12	1	0	0	0	Caudal fin rot	1	Caudal fin rot /skin ulcers	2	Caudal fin rot /skin ulcers	0	0
	YO19	5	0	0	0	Caudal fin rot	0	Caudal fin rot	0	Caudal fin rot	0	0
	YO51	3	0	0	0	Caudal fin rot	1	Caudal fin rot	0	Caudal fin rot	0	0
	YO60	2	0	0	0	Caudal fin rot	1	Caudal fin rot	2	Caudal fin rot	0	0
	YO64	4	0	0	0	Caudal fin rot	1	Caudal fin rot	0	Caudal fin rot	0	0
	Control	6	0	0	0	Caudal fin rot	0	Caudal fin rot	0	Caudal fin rot	0	0
3	YO12	1	0	0	0	Caudal fin rot	0	Caudal fin rot	0	Caudal fin rot	0	0
	YO19	5	0	0	0	Caudal fin rot	0	Caudal fin rot	0	Caudal fin rot	0	0
	YO51	3	0	0	0	Caudal fin rot	0	Caudal fin rot	0	Caudal fin rot	0	0
	YO60	2	0	0	0	Caudal fin rot	0	Caudal fin rot	0	Caudal fin rot	0	0
	YO64	4	0	0	0	Caudal fin rot	0	Caudal fin rot	0	Caudal fin rot	0	0
	Control	6	0	0	0	Caudal fin rot	0	Caudal fin rot	0	Caudal fin rot	0	0



FIG. 5.1. Caudal fin rot observed in *Oreochromis mossambicus* after 72 h of exposure to isolate YO60 following severe handling stress.



FIG. 5.2. Skin ulceration observed in *Oreochromis mossambicus* after 96 h of exposure to isolate YO60 following severe handling stress.

The highest mortality occurred in the first 24 h, which varied from 30% (control) to 60% (isolate YO60) of the total number of fish exposed in each tank, respectively. After a decrease in mortality, the mortality rate suddenly increased in all the tanks after 120 h of exposure.

A similar pattern was observed in experiment 2, except no mortality was observed within the first 72 h. Earlier onset of symptoms was observed and fish mortality was only observed after 96 h of exposure. Highest mortality was observed in fish exposed to isolates YO12 and YO60. In experiment 3, earlier onset of symptoms was also observed, although only fish exposed to isolates YO12, YO51 and YO60 displayed caudal fin rot. No symptoms or mortality were recorded in the control tanks of both experiments 2 and 3. Twenty four hour temperature readings revealed an average temperature of 15°C in experiment 1, and 17°C in both experiments 2 and 3.

All the sampled fish from experiment 1 and 2 produced orange/yellow pigmented colonies with spreading nature, morphologically resembling *Flavobacterium* spp. Bacterial colonies morphologically resembling *Flavobacterium* spp. predominated in all the samples taken from tanks 1 (YO19), 2 (YO64), 4 (YO60), 5 (YO51) and 6 (YO12), respectively, in experiment 1 and tanks 1 (YO12), 2 (YO60), 3 (YO51), and 4 (YO64), respectively, in experiment 2. Only two colonies morphologically resembling *Flavobacterium* spp., isolated from a spleen and liver sample, respectively, were obtained from sampled fish of tank 3 in experiment 1. Water samples taken after 10 days revealed the presence of *Flavobacterium* spp. in tanks, at much lower concentrations compared to the initial inoculum, which were initially inoculated with the selected *Flavobacterium* spp. isolates. Water samples from control tanks in all three experiments did not produce any colonies morphologically resembling *Flavobacterium* spp. Samples of the 10 randomly selected fish, prior to the infection study, taken from the gills, fins, liver and the spleen revealed the absence of *Flavobacterium* spp.

5.3.2 RAPD-PCR Fingerprint Analysis

RAPD fingerprints were obtained on amplification of all the re-isolated *Flavobacterium* spp. DNA (Fig. 5.3). On comparison of original RAPD profiles from stock cultures with profiles obtained from re-isolated flavobacteria, it was possible to successfully identify the bacterial isolates used in specific tanks. The isolate obtained from the spleen sample taken from tank 3 (control) produced a RAPD fingerprint identical to that of isolate YO19, however, the isolate obtained from the liver sample exhibited a unique RAPD fingerprint, suggesting an isolate not previously identified in the present study.

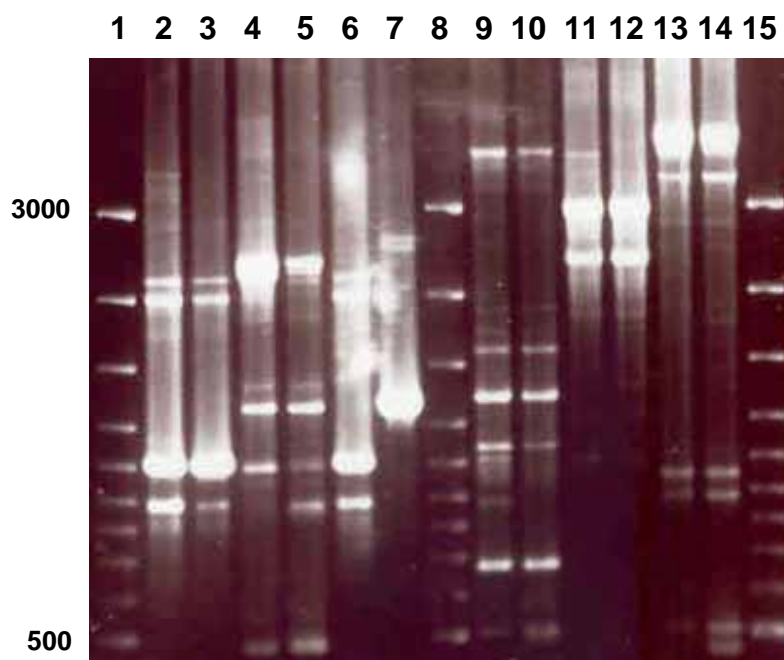


FIG. 5.3. Agarose gel electrophoresis of amplification products from *Flavobacterium* spp. DNA with the UBC 214 primer. Lanes 1, 8, and 15: O'GeneRuler 100 bp DNA ladder [Fermentas, Canada (3000/2000/1500/1200/1031/900/800/700/600/500/400/300/200/100 bp)]; Lanes 2-7: isolates YO19 (original culture), YO19 (re-isolated from infected fish), YO64 (original culture), YO64 (re-isolated from infected fish), spleen sample from tank 3 (fingerprint identical to isolate YO19), and liver sample from tank 3 (unique fingerprint), respectively, and lanes 9-14: isolates YO60 (original culture), YO60 (re-isolated from infected fish), YO51 (original culture), YO51 (re-isolated from infected fish), YO12 (original culture), and YO12 (re-isolated from infected fish), respectively.

5.4 DISCUSSION

Aquaculture as a means of food production is rapidly growing in South Africa. Many aquaculture systems are stunted due to severe disease outbreaks in farmed fish leading to high mortality, as local aquaculture farmers possess limited knowledge in terms of fish pathogens, disease symptoms and subsequent diagnosis in the SA setting. In the present study, the pathogenesis of the *Flavobacterium* spp. study isolates, to temperate water farmed fish has been demonstrated.

The presence of specific pathogens, stressful events and variations in temperature have been implicated in influencing the onset, incidence and severity of fish diseases (Dror *et al.*, 2005). Natural and artificial stress factors impair the homeostatic equilibrium in fish and affect physiological and immune functions (Dror *et al.*, 2005). Handling and confinement stress exerted on farmed fish has been shown to suppress the innate and adaptive immunity (Dror *et al.*, 2005). These stress factors have been suggested to predispose fish to infections (Dror *et al.*, 2005). Dror *et al.* (2005) noticed experimental handling stress applied to goldfish revealed gradually increasing breaches in integrity of gills and skin and the incidence of damage paralleled the level of stress. Additionally, fish infected after a double stress showed higher susceptibility to *A. salmonicida* infection and provided evidence that handling stress created additional ports of entry for pathogens, facilitating onset of disease (Dror *et al.*, 2005).

The tilapia used in experimental bath infections in the present study showed that the degree of handling stress was a major factor in determining the susceptibility to *Flavobacterium* spp. infection. In experiment 1, severe handling stress, based on a double stress, caused high mortality in fish. As described by Dror *et al.* (2005), it is likely that handling stress led to ports of entry for fish pathogens, including *Flavobacterium* spp., facilitating the onset of disease. This may in part explain the high mortality rate observed in the control tank, as infection caused by other fish pathogens was also facilitated.

Overall, isolates YO12, YO51 and YO60 showed the highest mortality and/or morbidity in all three experiments. Isolates YO12 and YO60 appeared to be the most virulent isolates, causing the death of 14 and 18 fish,

respectively, within the first 24 h and total mortality within 7 days in experiment 1 and the death of 3 fish each over the 10-day time period in experiment 2. These isolates also showed strong proteolytic activity for casein, however, low gelatin hydrolysis was observed by isolate YO60 and this may not play a role in determining virulence (Chapter 2, Table 2.2).

Isolates YO51 and YO64 also showed a high level of virulence in highly stressed fish, both resulting in the death of 13 fish within the first 24 h of experiment 1 and total mortality by isolate YO64 was observed within 8 days following infection. However, in experiment 2, only one fish died following exposure to isolate YO64 inoculum and no symptoms were exhibited by isolate YO64 infection in experiment 3, indicating possible weak virulence. Additionally, isolate YO51 also showed strong proteolytic activity for casein, whereas isolate YO64 showed weak proteolytic activity for casein (Chapter 2).

Isolates YO12, YO51 and YO60 were the only isolates responsible for the onset of symptoms of disease in fish in experiment 3. Isolate YO19 appeared to be the least virulent of all the isolates, although total mortality was observed within 8 days following infection in experiment 1. As observed for isolate YO64, isolate YO19 exhibited the smooth colony morphology type and showed weak proteolytic activity for casein. Colony morphology, based on the hazy and smooth colony morphology types, varied among these *Flavobacterium* spp. isolates and could not be specifically linked to virulence. However, most of the isolates exhibiting a high level of virulence shared the hazy colony morphology type and showed strong proteolytic activity for casein. Hence, a possible correlation between the ability to degrade casein, colony morphology type and virulence may exist. The onset of disease symptoms and fish mortality rate could not be linked to differences in temperature recorded in each respective experiment and thus infection caused by these *Flavobacterium* spp. isolates seemed non-temperature dependant. Bacteria such as *Aeromonas* spp. and *Pseudomonas* spp. were isolated to a far greater extent from the control tank (tank 3) of experiment 1, than from tanks seeded with the *Flavobacterium* spp. study isolates and may explain the variable results obtained in experiment 1 compared to experiments 2 and 3, for the respective *Flavobacterium* spp. study isolates.

According to 16S rRNA gene sequence analysis, isolates YO19 and YO64, appearing weakly virulent, distinctly clustered at 99% sequence similarity, whereas isolates YO51 and YO60, showing a higher level of virulence, formed separate clusters, showing variable degrees of sequence similarity (Chapter 3, Figs. 3.2-3.3). This may be a possible indication of strain-specific virulence phenotypes. Neither distinct RAPD and REP profiles, nor the presence/absence of the small plasmid could be specifically linked to virulence.

Isolates YO12, YO51 and YO60, showing a high level of virulence, exhibited WCP profiles A1, C, and A2, respectively, and isolate YO19 and YO64, least or less virulent, exhibited WCP profile B. Hence, isolates exhibiting protein profile B may be less virulent and a possible correlation between WCP profiles and virulence may exist. Additionally, bands of approximately ~65 kDa and ~27 kDa were shared by isolates exhibiting a high level of virulence (Chapter 3, Table 3.5). The ~65 kDa protein was the same size as the *F. psychrophilum* membrane-associated glycoprotein P60 (Merle *et al.*, 2003), which may facilitate bacterial attachment to host tissue, aiding in virulence of *Flavobacterium* spp. A lectin-like substance, potentially representing a glycoprotein, was shown to aid in attachment to gill tissue of a highly virulent *F. columnare* strain (Decostere *et al.*, 1999a). Glycoproteins are classified as S-layer proteins, which are known to aid in virulence (Sara and Sleytr, 2000). Although an OMP of similar size was present in OMP profiles of the *Flavobacterium* spp. isolates, no correlation could be established between virulence and the profiles exhibiting this OMP.

Flavobacterium spp. study isolates exhibiting WCP profile B were found to show strong biofilm formation in both microtiter-plate adherence assays and flow cell studies. This suggested a lack of correlation between a high degree of virulence and strong biofilm-forming capability existed. In contrast, it appeared less virulent isolates displayed a strong degree of adherence. The proteins involved in bacterial attachment to solid supports and to fish host tissue, such as the gills, may not necessarily be the same. Decostere *et al.* (1999a) showed the lectin-like substance mediating *F. columnare* attachment to gill tissue had an affinity for carbohydrate receptors.

The proteins observed in WCP profiles of virulent isolates may have other specific receptors, which mediate attachment to biotic or abiotic surfaces. Further investigation is needed to identify these proteins and to establish possible correlations between protein presence and virulence.

The tilapia used as a model of temperate freshwater fish appeared to recover rather quickly from disease symptoms in experiments 2 and 3, and showed significantly lower mortality and/or morbidity when moderately stressed, compared to highly stressed fish in experiment 1. Different fish species showing different levels of susceptibility to the same fish pathogens have been reported (Figueiredo *et al.*, 2005; and Stringer-Roth *et al.*, 2002). Figueiredo *et al.* (2005) found differences in susceptibility of Nile tilapia and channel catfish to experimental *F. columnare* infection. Nile tilapia showed significant lower susceptibility to *F. columnare*, which may be related to differences in resistance and immune status between the fish species (Figueiredo *et al.*, 2005). Differences in susceptibility to *F. columnare* have also been shown in experimental bath infections of barramundi and goldfish, with the latter being less susceptible, showing lower mortality (Soltani *et al.*, 1996 – cited by Stringer-Roth *et al.*, 2002). Hence, the use of tilapia as a model of temperate freshwater fish species may not be ideal in *Flavobacterium* virulence studies. Due to unforeseen unfortunate circumstances, it was not possible to test rainbow trout in the present study.

Additionally, the *Flavobacterium* spp. isolates used in experimental infections were isolated from diverse fish species (Chapter 2, Table 2.1), other than tilapia. Isolates YO12, YO19 and YO51 were obtained from diseased rainbow trout, exhibiting severe skin lesions, fin rot and gill necrosis, whereas isolates YO60 and YO64 were isolated from longfin eel, exhibiting only severe gill necrosis. Symptoms established in *Flavobacterium* infection of tilapia included skin ulceration, fin rot and gill necrosis (Figs. 5.1-5.2). All the isolates were re-isolated from internal organs, indicative of septicemic disease in experiments 1 and 2. Ekman (2003) has reported differences in pathology of *F. psychrophilum* in different salmonid species. Infected rainbow trout showed more pronounced lesions in the spleen compared to symptoms displayed by Atlantic salmon and sea trout (Ekman, 2003). Similarly, differences in symptoms were observed in the tilapia, compared to the original

host species the *Flavobacterium* spp. isolates were isolated from. However, the original isolation of these *Flavobacterium* spp. isolates from diverse fish species, is proof of its pathogenicity in a wide range of fish species.

Although no reliable protocol exists for reproducible experimental fish infections, the experimental infection model, incorporating handling stress as a predisposing susceptibility factor, used in this study clearly indicated the importance of stress-related factors in fish infection. This is in accordance with findings reported by Dror *et al.* (2005) who showed experimental handling stress, simulating an aquaculture stressor, significantly enhanced the rate of fish infection. Hence, the use of stressors in experimental fish infections, regarding virulence of fish pathogens in farmed fish, may be used as standard protocol in future infection studies.

The RAPD-PCR fingerprinting technique was a rapid means of screening the re-isolated *Flavobacterium* spp. isolates. This technique was successfully used to identify all the *Flavobacterium* spp. isolates, which produced identical fingerprints when compared to RAPD fingerprints from original *Flavobacterium* spp. study isolates (Fig. 5.3). This confirmed the reproducibility index of 100% and proved the excellent discriminatory power of this technique. The isolate, obtained from the control tank spleen sample in experiment 1, showing an identical RAPD fingerprint to that of isolate YO19 (Fig. 5.3), was likely to be a cross contaminant, as tank 1, containing the isolate YO19 inoculum, was adjacent to the control tank 3. The unidentified isolate obtained from the control tank liver sample, showing a unique RAPD fingerprint, suggested that a pool of genetically heterogeneous *Flavobacterium* spp. may be circulating in fish aquaculture systems.

The use of the RAPD-PCR technique is recommended for future epidemiological typing of *Flavobacterium* spp. isolates in South Africa. This typing technique may be used as an effective and rapid diagnostic tool to identify specific types of *Flavobacterium* spp. isolated from diseased fish and may lead to their successful treatment and control. This RAPD technique may also be effective in differentiating among other fish pathogenic *Flavobacterium* spp., including *F. columnare* and *F. psychrophilum*, and further investigation is needed in this regard.

CHAPTER SIX

CONCLUDING REMARKS

6.1 THE RESEARCH IN PERSPECTIVE

In the present study, *Flavobacterium* spp. isolated from a variety of fish species in South Africa were identified and characterized by a combination of phenotypic and biochemical methods. The use of molecular typing methods for the rapid identification and possible diagnosis of *Flavobacterium* spp. isolates from fish disease outbreaks was also investigated. Investigation of biofilm formation by *Flavobacterium* spp. and possible virulence factors was also assessed. This is the first reported isolation and characterisation of *Flavobacterium* spp. from diseased fish in South Africa.

Phenotypic, physiological and biochemical characterisation allowed limited discrimination of the 32 *Flavobacterium* spp. isolates. This is in accordance with other studies based on the identification and characterisation of *Flavobacterium* spp., especially studies regarding *F. columnare* (Bernardet *et al.*, 1996; Beranardet *et al.*, 2002; and Triyanto and Wakabayashi, 1999). However, these methods are essential for preliminary identification of *Flavobacterium* spp. isolates to the genus level, and were thus useful in the current study (Bernardet and Bowman, 2005; Bernardet *et al.*, 1996; and Bernardet *et al.*, 2002).

16S rRNA gene sequence analysis revealed the high degree ($\geq 97\%$) of similarity between the *Flavobacterium* spp. isolates and *F. johnsoniae*, respectively. It is interesting to note the strong degree of similarity shared by *Flavobacterium* spp. study isolates to sequences from a rainbow trout intestinal bacterium and that from an isolate from a river in Southern Germany (Bohnert *et al.*, 2001; and Huber *et al.*, 2004). *F. johnsoniae*-like flavobacteria appear to be significantly more important in the Southern African context as aquaculture pathogens than *F. columnare* and *F. psychrophilum* in the USA, Europe and South America (Bernardet and Bowman, 2005). This technique is recommended by Bernardet *et al.* (2002) as a tool for the identification of *Flavobacterium* species at the genus level. However, it should be used in

combination with other genomic studies, including DNA-DNA hybridization studies, as it is not specific enough to identify *Flavobacterium* spp. to the specific level (Bernardet *et al.*, 2002). This was also the case in the present study and although high 16S rRNA gene similarity was shared with *F. johnsoniae*, *Flavobacterium* spp. isolates could not be classified as *F. johnsoniae*, as the cutoff value for species level is estimated at 97% 16S rRNA gene sequence similarity (Bernardet and Bowman, 2005; and Bernardet *et al.*, 2002). However, this technique proved to be useful for determining phylogenetic relationships with members of the genus *Flavobacterium*.

Although 16S rRNA gene PCR-RFLP discrimination was limited, molecular typing of the *Flavobacterium* spp. isolates using RAPD fingerprinting, REP fingerprinting, WCP analysis and OMP analysis, provided evidence of the genetic heterogeneity among closely related *Flavobacterium* spp. isolates. The genetic heterogeneity among *F. columnare* strains is well documented (Bernardet and Bowman, 2005; Bernardet *et al.*, 1996; Bernardet *et al.*, 2002; and Triyanto and Wakabayashi, 1999). The poor discrimination of the *Flavobacterium* spp. study isolates by 16S rRNA gene PCR-RFLP analysis is in accordance with Triyanto and Wakabayashi (1999), although it has been useful for the classification of *F. columnare* strains into three distinct genomovars. This technique, in combination with 16S rRNA gene sequence analysis, however, ruled out the possibility of *Flavobacterium* spp. isolates in the present study being identified as *F. columnare* strains.

Similar RAPD-PCR and REP-PCR profiles were indicative of a possible clonal origin of groups of *Flavobacterium* spp. isolates, although a number of these isolates had been isolated from different fish species and geographical locations. Several genetic markers were observed in both RAPD and REP profiles, which could be useful for potential diagnostic application or direct identification purposes. A high degree of genetic heterogeneity in the *Flavobacterium* spp. isolates was observed using molecular techniques [Discrimination indices of 0.9194 (RAPD-PCR), 0.9516 (REP-PCR), and 0.9458 (OMP analysis)], although certain isolates appeared to be identical, or closely related. A correlation between RAPD types and fish host species has been reported (Chakroun *et al.*, 1998), although this was not observed in the

current study. No clear relationship was observed between RAPD or REP results and 16S rRNA gene RFLP results.

Four WCP profiles were obtained for the *Flavobacterium* spp. isolates tested and this technique showed limited discrimination of the *Flavobacterium* spp. study isolates. Eighteen OMP profiles were obtained for these *Flavobacterium* spp. isolates and could in certain instances be correlated with RAPD and REP profiles, suggesting clonal origins. Dominant clones were observed in the *Flavobacterium* spp. isolates obtained from rainbow trout and eel, respectively, which may serve as dominant fish host species or carriers for these clones. WCP and OMP profiles showed the presence of both unique and common proteins, which may differentiate *Flavobacterium* species or strains, or may potentially serve for vaccine development.

The biofilm-forming ability of *Flavobacterium* spp. isolates was confirmed with the use of both the microtiter-plate adherence assay and flow cell studies. Both techniques effectively differentiated strong and weakly adherent isolates. The modified Stepanovic *et al.* (2000) microtiter-plate adherence assay was a rapid and highly reproducible technique for screening a large group of isolates. This quantitative assay is superior in terms of objectivity, accuracy and reproducibility compared to the quantitative assay initially used. Although the flow cell study was more time-consuming, important qualitative information about the biofilm structures produced by *Flavobacterium* spp. isolates was obtained. Strong correlation was observed between strong adherence and the specific WCP profile (profile B) indicating that specific isolates have stronger biofilm phenotypes than other *Flavobacterium* spp. isolates.

Investigation of virulence using handling related stress as a facilitating factor in an infection study proved the pathogenicity of *Flavobacterium* spp. isolates in tilapia in the presence of a stressor predisposing to infection. This is in accordance with Dror *et al.* (2005) who showed handling stress breaches the skin and gill integrity, facilitating infection. No distinct correlation was established between biofilm formation and virulence of *Flavobacterium* spp. isolates, since, weakly virulent isolates showed strong adherence (biofilm formation), and vice versa.

The combination of phenotypic, biochemical and genomic studies is essential for identification and description of newly isolated members of the genus *Flavobacterium*. Phylogenetic analysis strictly using 16S rRNA gene sequencing alone is not effective in the classification of *Flavobacterium* spp. isolates at the species level and a more polyphasic approach is crucial. Phenotypic and molecular typing techniques are useful for studying the genetic diversity of *Flavobacterium* spp. and reveal important information on the clonality of *Flavobacterium* spp. isolated from diverse fish species and geographical regions. Genetic typing methods provide a clearer understanding of the extent of genetic heterogeneity within populations of *Flavobacterium* spp. isolates.

Quantitative adherence assays are rapid techniques for classification of the biofilm-forming abilities of bacteria, including *Flavobacterium* spp. The use of flow cell studies can expand *Flavobacterium* biofilm knowledge on a qualitative basis and reveal important visual characteristics of biofilm structure and cell-cell interaction between flavobacteria and different bacterial species, respectively.

Infection experiments may be useful for identification of virulent *Flavobacterium* spp. strains. Incorporation of stress factors commonly occurring in aquaculture greatly enhances the infecting ability of pathogenic organisms, including fish pathogenic *Flavobacterium* spp., and plays a crucial role in infection protocols. The combination of typing systems and virulence studies may effectively be used in epidemiological studies to distinguish among virulent and non-virulent *Flavobacterium* spp. strains. This would facilitate the use of effective control measures for *Flavobacterium* disease outbreaks and the ultimate prevention of high fish mortality in aquaculture.

Future work will entail understanding the molecular basis of biofilm formation. This will entail investigating the role of genes involved in the gliding motility phenotype. Additionally, it will be necessary to investigate differential expression of proteins in planktonic and sessile flavobacterial cells in order to understand the triggers and consequences of biofilm formation.

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APPENDIX 1

YO10GAACGCTAGCGGCAGGCTTAACACATGCAA	30
YO11GGATGAACGCTAGCGGCAGGCTTAACACATGCAA	34
YO15ACGCTG.CGGCTNNCTTA.CACCTGCNA	26
YO19CGGCAGGCTTACACATGCAA	20
YO34GACNCTNCGGCTNGCTTACACATGCA	27
YO46CNCTA.CGGCNG.CTTA.C.CATGCNA	23
YO51GNACGCTNACGGCCCCCTTTACACATGCAA	30
YO60CGGC.CGGCTTAACACATGCAA	21
YO64TGCAA	5
FJOHNSONIAE	ACGATGAAGAGTTTGATCCTGGCTCAGGATNAACGCTAGCGGCAGGCTTAACACATGCAA	60
FRAINBOWTROUGAGTTTGATTATGGCTCAGGATGAACGCTAGCGGCAGGCTTAACACATGCAA	52
FWUBA46ACGCTAGCGGCAGGCTTAACACATGCAA	28
YO10	GTCGAGGGGTATATGCTCTTCGGATATAGAGACCGGCGCACGGGTGCGTAACGCGTATGCA	90
YO11	GTCGAGGGGTATATGCTCTTCGGATATAGAGACCGGCGCACGGGTGCGTAACGCGTATGCA	94
YO15	GTCGAGGGGTATATGCTCTTCGGATATAGAGACCGGCGCACGGGTGCGTAACGCGTATGCA	86
YO19	GTCGAGGGGTATATGCTCTTCGGATATAGAGACCGGCGCACGGGTGCGTAACGCGTATGCA	80
YO34	GTCGAGGGGTATATGCTCTTCGGATATAGAGACCGGCGCACGGGTGCGTAACGCGTATGCA	87
YO46	GTCGAGGGGTATATGCTCTTCGGATATAGAGACCGGCGCACGGGTGCGTAACGCGTATGCA	83
YO51	GTCGAGGGGTATATGCTCTTCGGAGCTAGAGACCGGCGCACGGGTGCGTAACGCGTATGCA	90
YO60	GTCGAGGGGTATGCTTCTTCGGAAGCAGAGACCGGCGCACGGGTGCGTAACGCGTATGCA	81
YO64	GTCGAGGGGTATATGCTCTTCGGATATAGAGACCGGCGCACGGGTGCGTAACGCGTATGCA	65
FJOHNSONIAE	GTCGAGGGGTATACTTCTTCGGAAGTAGAGACCGGCGCACGGGTGCGTAACGCGTATGCA	120
FRAINBOWTROU	GTCGAGGGGTATATGCTCTTCGGAGCTAGAGACCGGCGCACGGGTGCGTAACGCGTATGCA	112
FWUBA46	GTCGAGGGGTATGCTTCTTCGGAGCAGAGACCGGCGCACGGGTGCGTAACGCGTATGCA	88
YO10	ATCTACCTTTTACAGAGGGATAGCCCAGAGAAATTTGGATTAATACCTCATAGTATAGTG	150
YO11	ATCTACCTTTTACAGAGGGATAGCCCAGAGAAATTTGGATTAATACCTCATAGTATAGTG	154
YO15	ATCTACCTTTTACAGAGGGATAGCCCAGAGAAATTTGGATTAATACCTCATAGTATAGTG	146
YO19	ATCTACCTTTTACAGAGGGATAGCCCAGAGAAATTTGGATTAATACCTCATAGTATATTT	140
YO34	ATCTACCTTTTACAGAGGGATAGCCCAGAGAAATTTGGATTAATACCTCATAGTATATTT	147
YO46	ATCTACCTTTTACAGAGGGATAGCCCAGAGAAATTTGGATTAATACCTCATAGTATAGTG	143
YO51	ATCTACCTTTTACAGAGGGATAGCCCAGAGAAATTTGGATTAATACCTCATAGTATATTT	150
YO60	ATCTACCTTTTACAGAGGGATAGCCCAGAGAAATTTGGATTAATACCTCATAGTATAGCT	141
YO64	ATCTACCTTTTACAGAGGGATAGCCCAGAGAAATTTGGATTAATACCTCATAGTATATTT	125
FJOHNSONIAE	ATCTACCTTTTACAGAGGGATAGCCCAGAGAAATTTGGATTAATACCTCATAGTATATAG	180
FRAINBOWTROU	ATCTACCTTTTACAGAGGGATAGCCCAGAGAAATTTGGATTAATACCTCATAGTATATG	172
FWUBA46	ATCTACCTTTTACAGAGGGATAGCCCAGAGAAATTTGGATTAATACCTCATAGTATATAG	148
YO10	ACTCGGCATCGAGATACTATTAAAGTCACAACGGTAAAAGATGAGCATGCGTCCCATTAG	210
YO11	ACTCGGCATCGAGATACTATTAAAGTCACAACGGTAAAAGATGAGCATGCGTCCCATTAG	214
YO15	ACTCGGCATCGAGATACTATTAAAGTCACAACGGTAAAAGATGAGCATGCGTCCCATTAG	206
YO19	GAGTGGCATCACTTAATTAATTAAGTCACAACGGTAAAAGATGAGCATGCGTCCCATTAG	200
YO34	GAGTGGCATCACTTAATTAATTAAGTCACAACGGTAAAAGATGAGCATGCGTCCCATTAG	207
YO46	ACTCGGCATCGAGATACTATTAAAGTCACAACGGTAAAAGATGAGCATGCGTCCCATTAG	203
YO51	GNGTGGCATCACTTAATTAATTAAGTCACAACGGTAAAAGATGAGCATGCGTCCCATTAG	210
YO60	GAATCGCATGATTCTACTATTAAAGTCACAACGGTAAAAGATGAGCATGCGTCCCATTAG	201
YO64	GAGTGGCATCACTTAATTAATTAAGTCACAACGGTAAAAGATGAGCATGCGTCCCATTAG	185
FJOHNSONIAE	ACTCGGCATCGAGATTATATTAAAGTCACAACGGTAAAAGATGAGCATGCGTCCCATTAG	240
FRAINBOWTROU	AAATGCGATCATTTTATTAATTAAGTCACAACGGTAAAAGATGAGCATGCGTCCCATTAG	232
FWUBA46	ACTCGGCATCGAGATTATATTAAAGTCACAACGGTAAAAGATGAGCATGCGTCCCATTAG	208

YO10	CTAGTTGGTAAGGTAACGGCTTACCAAGGCTACGATGGGTAGGGGTCTGAGAGGGAGAT	270
YO11	CTAGTTGGTAAGGTAACGGCTTACCAAGGCTACGATGGGTAGGGGTCTGAGAGGGAGAT	274
YO15	CTAGTTGGTAAGGTAACGGCTTACCAAGGCTACGATGGGTAGGGGTCTGAGAGGGAGAT	266
YO19	CTAGTTGGTAAGGTAACGGCTTACCAAGGCTACGATGGGTAGGGGTCTGAGAGGGAGAT	260
YO34	CTAGTTGGTAAGGTAACGGCTTACCAAGGCTACGATGGGTAGGGGTCTGAGAGGGAGAT	267
YO46	CTAGTTGGTAAGGTAACGGCTTACCAAGGCTACGATGGGTAGGGGTCTGAGAGGGAGAT	263
YO51	CTAGTTGGTAAGGTAACGGCTTACCAAGGCTACGATGGGTAGGGGTCTGAGAGGGAGAT	270
YO60	CTAGTTGGTAAGGTAACGGCTTACCAAGGCTACGATGGGTAGGGGTCTGAGAGGGAGAT	261
YO64	CTAGTTGGTAAGGTAACGGCTTACCAAGGCTACGATGGGTAGGGGTCTGAGAGGGAGAT	245
FJOHNSONIAE	CTAGTTGGTAAGGTAACGGCTTACCAAGGCTACGATGGGTAGGGGTCTGAGAGGGAGAT	300
FRAINBOWTROU	CTAGTTGGTAAGGTAACGGCTTACCAAGGCTACGATGGGTAGGGGTCTGAGAGGGAGAT	292
FWUBA46	CTAGTTGGTAAGGTAACGGCTTACCAAGGCTACGATGGGTAGGGGTCTGAGAGGGAGAT	268
YO10	CCCCCAGACTGGTACTGAGACACGGACCAGACTCCTACGGGAGGCAGCAGTGAGGAATAT	330
YO11	CCCCCAGACTGGTACTGAGACACGGACCAGACTCCTACGGGAGGCAGCAGTGAGGAATAT	334
YO15	CCCCCAGACTGGTACTGAGACACGGACCAGACTCCTACGGGAGGCAGCAGTGAGGAATAT	326
YO19	CCCCCAGACTGGTACTGAGACACGGACCAGACTCCTACGGGAGGCAGCAGTGAGGAATAT	320
YO34	CCCCCAGACTGGTACTGAGACACGGACCAGACTCCTACGGGAGGCAGCAGTGAGGAATAT	327
YO46	CCCCCAGACTGGTACTGAGACACGGACCAGACTCCTACGGGAGGCAGCAGTGAGGAATAT	323
YO51	CCCCCAGACTGGTACTGAGACACGGACCAGACTCCTACGGGAGGCAGCAGTGAGGAATAT	330
YO60	CCCCCAGACTGGTACTGAGACACGGACCAGACTCCTACGGGAGGCAGCAGTGAGGAATAT	321
YO64	CCCCCAGACTGGTACTGAGACACGGACCAGACTCCTACGGGAGGCAGCAGTGAGGAATAT	305
FJOHNSONIAE	CCCCCAGACTGGTACTGAGACACGGACCAGACTCCTACGGGAGGCAGCAGTGAGGAATAT	360
FRAINBOWTROU	CCCCCAGACTGGTACTGAGACACGGACCAGACTCCTACGGGAGGCAGCAGTGAGGAATAT	352
FWUBA46	CCCCCAGACTGGTACTGAGACACGGACCAGACTCCTACGGGAGGCAGCAGTGAGGAATAT	328
YO10	TGGACAATGGGCGCAAGCCTGATCCAGCCATGCCGCGTGCAGGATGACGGTCCTATGGAT	390
YO11	TGGACAATGGGCGCAAGCCTGATCCAGCCATGCCGCGTGCAGGATGACGGTCCTATGGAT	394
YO15	TGGACAATGGGCGCAAGCCTGATCCAGCCATGCCGCGTGCAGGATGACGGTCCTATGGAT	386
YO19	TGGACAATGGGCGCAAGCCTGATCCAGCCATGCCGCGTGCAGGATGACGGTCCTATGGAT	380
YO34	TGGACAATGGGCGCAAGCCTGATCCAGCCATGCCGCGTGCAGGATGACGGTCCTATGGAT	387
YO46	TGGACAATGGGCGCAAGCCTGATCCAGCCATGCCGCGTGCAGGATGACGGTCCTATGGAT	383
YO51	TGGACAATGGGCGCAAGCCTGATCCAGCCATGCCGCGTGCAGGATGACGGTCCTATGGAT	390
YO60	TGGACAATGGGCGCAAGCCTGATCCAGCCATGCCGCGTGCAGGATGACGGTCCTATGGAT	381
YO64	TGGACAATGGGCGCAAGCCTGATCCAGCCATGCCGCGTGCAGGATGACGGTCCTATGGAT	365
FJOHNSONIAE	TGGACAATGGGCGCAAGCCTGATCCAGCCATGCCGCGTGCAGGATGACGGTCCTATGGAT	420
FRAINBOWTROU	TGGACAATGGGCGCAAGCCTGATCCAGCCATGCCGCGTGCAGGATGACGGTCCTATGGAT	412
FWUBA46	TGGACAATGGGCGCAAGCCTGATCCAGCCATGCCGCGTGCAGGATGACGGTCCTATGGAT	388
YO10	TGTAAACTGCTTTTGTACGAGAAGAAACACTCCTACGTGTAGGAGCTTGACGGTATCGTA	450
YO11	TGTAAACTGCTTTTGTACGAGAAGAAACACTCCTACGTGTAGGAGCTTGACGGTATCGTA	454
YO15	TGTAAACTGCTTTTGTACGAGAAGAAACACTCCTACGTGTAGGAGCTTGACGGTATCGTA	446
YO19	TGTAAACTGCTTTTGTACGAGAAGAAACACTGCTTCGTGAAGCAGCTTGACGGTATCGTA	440
YO34	TGTAAACTGCTTTTGTACGAGAAGAAACACTGCTTCGTGAAGCAGCTTGACGGTATCGTA	447
YO46	TGTAAACTGCTTTTGTACGAGAAGAAACACTCCTTCGTGAAGCAGCTTGACGGTATCGTA	443
YO51	TGTAAACTGCTTTTGTACGAGAAGAAACACTCCAACGTGTTGGAGCTTGACGGTATCGTA	450
YO60	TGTAAACTGCTTTTGTACGAGAAGAAACACTGCTTCGTGAAGCAGCTTGACGGTATCGTA	441
YO64	TGTAAACTGCTTTTGTACGAGAAGAAACACTGCTTCGTGAAGCAGCTTGACGGTATCGTA	425
FJOHNSONIAE	TGTAAACTGCTTTTGTACGAGAAGAAACACTGCTACGTGTAGGAGCTTGACGGTATCGTA	480
FRAINBOWTROU	TGTAAACTGCTTTTGTACGAGAAGAAACACTCCGACGTGTCGGAGCTTGACGGTATCGTA	472
FWUBA46	TGTAAACTGCTTTTGTACGAGAAGAAACACTGGTTCGTGAAGCAGCTTGACGGTATCGTA	448

Y010	AGAATAAGGATCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGATCCAGCGTT	510
Y011	AGAATAAGGATCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGATCCAGCGTT	514
Y015	AGAATAAGGATCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGATCCAGCGTT	506
Y019	AGAATAAGGATCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGATCCAGCGTT	500
Y034	AGAATAAGGATCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGATCCAGCGTT	507
Y046	AGAATAAGGATCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGATCCAGCGTT	503
Y051	AGAATAAGGATCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGATCCAGCGTT	510
Y060	AGAATAAGGATCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGATCCAGCGTT	501
Y064	AGAATAAGGATCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGATCCAGCGTT	485
FJOHNSONIAE	AGAATAAGGATCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGATCCAGCGTT	540
FRAINBOWTROU	AGAATAAGGATCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGATCCAGCGTT	532
FWUBA46	AGAATAAGGATCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGATCCAGCGTT	508
Y010	ATCCGGAATCATTGGGTTTAAAGGGTCCGTAGGCGGTTTAGTAAGTCAGTGGTGAAAGCC	570
Y011	ATCCGGAATCATTGGGTTTAAAGGGTCCGTAGGCGGTTTAGTAAGTCAGTGGTGAAAGCC	574
Y015	ATCCGGAATCATTGGGTTTAAAGGGTCCGTAGGCGGTTTAGTAAGTCAGTGGTGAAAGCC	566
Y019	ATCCGGAATCATTGGGTTTAAAGGGTCCGTAGGCGGTTTAGTAAGTCAGTGGTGAAAGCC	560
Y034	ATCCGGAATCATTGGGTTTAAAGGGTCCGTAGGCGGTTTAGTAAGTCAGTGGTGAAAGCC	567
Y046	ATCCGGAATCATTGGGTTTAAAGGGTCCGTAGGCGGTTTAGTAAGTCAGTGGTGAAAGCC	563
Y051	ATCCGGAATCATTGGGTTTAAAGGGTCCGTAGGCGGTTTAGTAAGTCAGTGGTGAAAGCC	570
Y060	ATCCGGAATCATTGGGTTTAAAGGGTCCGTAGGCGGTTTAGTAAGTCAGTGGTGAAAGCC	561
Y064	ATCCGGAATCATTGGGTTTAAAGGGTCCGTAGGCGGTTTAGTAAGTCAGTGGTGAAAGCC	545
FJOHNSONIAE	ATCCGGAATCATTGGGTTTAAAGGGTCCGTAGGCGGTTTAGTAAGTCAGTGGTGAAAGCC	600
FRAINBOWTROU	ATCCGGAATCATTGGGTTTAAAGGGTCCGTAGGCGGTTTAGTAAGTCAGTGGTGAAAGCC	592
FWUBA46	ATCCGGAATCATTGGGTTTAAAGGGTCCGTAGGCGGTTTAGTAAGTCAGTGGTGAAAGCC	568
Y010	CATCGCTCAACGGTGGAACGGCCATTGATACTGCTAAACTTGAATTATTTAGGAAGTAA	630
Y011	CATCGCTCAACGGTGGAACGGCCATTGATACTGCTAAACTTGAATTATTTAGGAAGTAA	632
Y015	CATCGCTCAACGGTGGAACGGCCATTGATACTGCTAAACTTGAATTATTTAGGAAGTAA	624
Y019	CATCGCTCAACGGTGGAACGGCCATTGATACTGCTAAACTTGAATTATTTAGGAAGTAA	618
Y034	CATCGCTCAACGGTGGAACGGCCATTGATACTGCTAAACTTGAATTATTTAGGAAGTAA	625
Y046	CATCGCTCAACGGTGGAACGGCCATTGATACTGCTAAACTTGAATTATTTAGGAAGTAA	621
Y051	CATCGCTCAACGGTGGAACGGCCATTGATACTGCTAAACTTGAATTATTTAGGAAGTAA	628
Y060	CATCGCTCAACGGTGGAACGGCCATTGATACTGCTAAACTTGAATTATTTAGGAAGTAA	619
Y064	CATCGCTCAACGGTGGAACGGCCATTGATACTGCTAAACTTGAATTATTTAGGAAGTAA	603
FJOHNSONIAE	CATCGCTCAACGGTGGAACGGCCATTGATACTGCTAAACTTGAATTATTTAGGAAGTAA	658
FRAINBOWTROU	CATCGCTCAACGGTGGAACGGCCATTGATACTGCTAAACTTGAATTATTTAGGAAGTAA	650
FWUBA46	CATCGCTCAACGGTGGAACGGCCATTGATACTGCTAAACTTGAATTATTTAGGAAGTAA	626
Y010	CTAGAATATGTAGTGTAACGGTGAAATGCTTAGAGATTACATGGAAATACCAATTGCGA	688
Y011	CTAGAATATGTAGTGTAACGGTGAAATGCTTAGAGATTACATGGAAATACCAATTGCGA	690
Y015	CTAGAATATGTAGTGTAACGGTGAAATGCTTAGAGATTACATGGAAATACCAATTGCGA	682
Y019	CTAGAATATGTAGTGTAACGGTGAAATGCTTAGAGATTACATGGAAATACCAATTGCGA	678
Y034	CTAGAATATGTAGTGTAACGGTGAAATGCTTAGAGATTACATGGAAATACCAATTGCGA	683
Y046	CTAGAATATGTAGTGTAACGGTGAAATGCTTAGAGATTACATGGAAATACCAATTGCGA	679
Y051	CTAGAATATGTAGTGTAACGGTGAAATGCTTAGAGATTACATGGAAATACCAATTGCGA	686
Y060	CTAGAATATGTAGTGTAACGGTGAAATGCTTAGAGATTACATGGAAATACCAATTGCGA	677
Y064	CTAGAATATGTAGTGTAACGGTGAAATGCTTAGAGATTACATGGAAATACCAATTGCGA	661
FJOHNSONIAE	CTAGAATATGTAGTGTAACGGTGAAATGCTTAGAGATTACATGGAAATACCAATTGCGA	716
FRAINBOWTROU	CTAGAATATGTAGTGTAACGGTGAAATGCTTAGAGATTACATGGAAATACCAATTGCGA	708
FWUBA46	CTAGAATATGTAGTGTAACGGTGAAATGCTTAGAGATTACATGGAAATACCAATTGCGA	684

YO10	AGGCAGGTTACTACTAATGGATTGACGCTGATGGACGAAAAGCGTGGGTAGCGAACAGGAT	748
YO11	AGGCAGGTTACTACTAATGGATTGACGCTGATGGACGAAAAGCGTGGGTAGCGAACAGGAT	750
YO15	AGGCAGGTTACTACTAATGGATTGACGCTGATGGACGAAAAGCGTGGGTAGCGAACAGGAT	742
YO19	AGGCAGGTTACTACTAATGGATTGACGCTGATGGACGAAAAGCGTGGGTAGCGAACAGGAT	738
YO34	AGGCAGGTTACTACTAATGGATTGACGCTGATGGACGAAAAGCGTGGGTAGCGAACAGGAT	743
YO46	AGGCAGGTTACTACTAATGGATTGACGCTGATGGACGAAAAGCGTGGGTAGCGAACAGGAT	739
YO51	AGGCAGGTTACTACTAATGGATTGACGCTGATGGACGAAAAGCGTGGGTAGCGAACAGGAT	746
YO60	AGGCAGGTTACTACTAATGGATTGACGCTGATGGACGAAAAGCGTGGGTAGCGAACAGGAT	737
YO64	AGGCAGGTTACTACTAATGGATTGACGCTGATGGACGAAAAGCGTGGGTAGCGAACAGGAT	721
FJOHNSONIAE	AGGCAGGTTACTACTAATGGATTGACGCTGATGGACGAAAAGCGTGGGTAGCGAACAGGAT	776
FRAINBOWTROU	AGGCAGGTTACTACTAATGGATTGACGCTGATGGACGAAAAGCGTGGGTAGCGAACAGGAT	768
FWUBA46	AGGCAGGTTACTACTAATGGATTGACGCTGATGGACGAAAAGCGTGGGTAGCGAACAGGAT	744
YO10	TAGATACCCTGGTAGTCCACGCCGTAAACGATGGATACTAGCTGTTGCAAGCAATTTTCAG	808
YO11	TAGATACCCTGGTAGTCCACGCCGTAAACGATGGATACTAGCTGTTGCAAGCAATTTTCAG	810
YO15	TAGATACCCTGGTAGTCCACGCCGTAAACGATGGATACTAGCTGTTGCAAGCAATTTTCAG	802
YO19	TAGATACCCTGGTAGTCCACGCCGTAAACGATGGATACTAGCTGTTGCAAGCAATTTTCAG	798
YO34	TAGATACCCTGGTAGTCCACGCCGTAAACGATGGATACTAGCTGTTGCAAGCAATTTTCAG	803
YO46	TAGATACCCTGGTAGTCCACGCCGTAAACGATGGATACTAGCTGTTGCAAGCAATTTTCAG	799
YO51	TAGATACCCTGGTAGTCCACGCCGTAAACGATGGATACTAGCTGTTGCAAGCAATTTTCAG	806
YO60	TAGATACCCTGGTAGTCCACGCCGTAAACGATGGATACTAGCTGTTGCAAGCAATTTTCAG	797
YO64	TAGATACCCTGGTAGTCCACGCCGTAAACGATGGATACTAGCTGTTGCAAGCAATTTTCAG	781
FJOHNSONIAE	TAGATACCCTGGTAGTCCACGCCGTAAACGATGGATACTAGCTGTTGCAAGCAATTTTCAG	836
FRAINBOWTROU	TAGATACCCTGGTAGTCCACGCCGTAAACGATGGATACTAGCTGTTGCAAGCAATTTTCAG	828
FWUBA46	TAGATACCCTGGTAGTCCACGCCGTAAACGATGGATACTAGCTGTTGCAAGCAATTTTCAG	804
YO10	TGGCTAAGCGAAAGTGATAAGTATCCACCTGGGGAGTACGTTTCGCAAGAATGAAACTCA	868
YO11	TGGCTAAGCGAAAGTGATAAGTATCCACCTGGGGAGTACGTTTCGCAAGAATGAAACTCA	870
YO15	TGGCTAAGCGAAAGTGATAAGTATCCACCTGGGGAGTACGTTTCGCAAGAATGAAACTCA	862
YO19	TGGCTAAGCGAAAGTGATAAGTATCCACCTGGGGAGTACGTTTCGCAAGAATGAAACTCA	858
YO34	TGGCTAAGCGAAAGTGATAAGTATCCACCTGGGGAGTACGTTTCGCAAGAATGAAACTCA	863
YO46	TGGCTAAGCGAAAGTGATAAGTATCCACCTGGGGAGTACGTTTCGCAAGAATGAAACTCA	859
YO51	TGGCTAAGCGAAAGTGATAAGTATCCACCTGGGGAGTACGTTTCGCAAGAATGAAACTCA	866
YO60	TGGCTAAGCGAAAGTGATAAGTATCCACCTGGGGAGTACGTTTCGCAAGAATGAAACTCA	857
YO64	TGGCTAAGCGAAAGTGATAAGTATCCACCTGGGGAGTACGTTTCGCAAGAATGAAACTCA	841
FJOHNSONIAE	TGGCTAAGCGAAAGTGATAAGTATCCACCTGGGGAGTACGTTTCGCAAGAATGAAACTCA	896
FRAINBOWTROU	TGGCTAAGCGAAAGTGATAAGTATCCACCTGGGGAGTACGTTTCGCAAGAATGAAACTCA	888
FWUBA46	TGGCTAAGCGAAAGTGATAAGTATCCACCTGGGGAGTACGTTTCGCAAGAATGAAACTCA	864
YO10	AAGGAATTGACGGGGGGCCCGCACAAAGCGGTGGAGCATGTGGTTTAATTTCGATGATACGCG	928
YO11	AAGGAATTGACGGGGGGCCCGCACAAAGCGGTGGAGCATGTGGTTTAATTTCGATGATACGCG	930
YO15	AAGGAATTGACGGGGGGCCCGCACAAAGCGGTGGAGCATGTGGTTTAATTTCGATGATACGCG	922
YO19	AAGGAATTGACGGGGGGCCCGCACAAAGCGGTGGAGCATGTGGTTTAATTTCGATGATACGCG	918
YO34	AAGGAATTGACGGGGGGCCCGCACAAAGCGGTGGAGCATGTGGTTTAATTTCGATGATACGCG	923
YO46	AAGGAATTGACGGGGGGCCCGCACAAAGCGGTGGAGCATGTGGTTTAATTTCGATGATACGCG	919
YO51	AAGGAATTGACGGGGGGCCCGCACAAAGCGGTGGAGCATGTGGTTTAATTTCGATGATACGCG	926
YO60	AAGGAATTGACGGGGGGCCCGCACAAAGCGGTGGAGCATGTGGTTTAATTTCGATGATACGCG	917
YO64	AAGGAATTGACGGGGGGCCCGCACAAAGCGGTGGAGCATGTGGTTTAATTTCGATGATACGCG	901
FJOHNSONIAE	AAGGAATTGACGGGGGGCCCGCACAAAGCGGTGGAGCATGTGGTTTAATTTCGATGATACGCG	956
FRAINBOWTROU	AAGGAATTGACGGGGGGCCCGCACAAAGCGGTGGAGCATGTGGTTTAATTTCGATGATACGCG	948
FWUBA46	AAGGAATTGACGGGGGGCCCGCACAAAGCGGTGGAGCATGTGGTTTAATTTCGATGATACGCG	924

YO10	AGGAACCTTACCAAGGCTTAAATGTAGTTTGACCGATTTGGAAACAGATCTTTCGCAAGA	988
YO11	AGGAACCTTACCAAGGCTTAAATGTAGTTTGACCGATTTGGAAACAGATCTTTCGCAAGA	990
YO15	AGGAACCTTACCAAGGCTTAAATGTAGTTTGACCGATTTGGAAACAGATCTTTCGCAAGA	982
YO19	AGGAACCTTACCAAGGCTTAAATGTAGATTGACCGCTTTGGAAACAGATCTTTCGCAAGA	978
YO34	AGGAACCTTACCAAGGCTTAAATGTAGATTGACCGCTTTGGAAACAGATCTTTCGCAAGA	983
YO46	AGGAACCTTACCAAGGCTTAAATGTAGTTTGACCGCTTTGGAAACAGATCTTTCGCAAGA	979
YO51	AGGAACCTTACCAAGGCTTAAATGTAGATTGACCGCTTTGGAAACAGATCTTTCGCAAGA	986
YO60	AGGAACCTTACCAAGGCTTAAATGTAGATTGACCGCTTTGGAAACAGATCTTTCGCAAGA	977
YO64	AGGAACCTTACCAAGGCTTAAATGTAGATTGACCGCTTTGGAAACAGATCTTTCGCAAGA	961
FJOHNSONIAE	AGGAACCTTACCAAGGCTTAAATGTAGATTGACCGCTTTGGAAACAGATCTTTCGCAAGA	1016
FRAINBOWTROU	AGGAACCTTACCAAGGCTTAAATGTAGATTGACCGCTTTGGAAACAGATCTTTCGCAAGA	1008
FWUBA46	AGGAACCTTACCAAGGCTTAAATGTAGATTGACCGCTTTGGAAACAGATCTTTCGCAAGA	984
YO10	CAAAATTACAAGGTGCTGCATGGTTGTCGTCAGCTCGTGCCGTGAGGTGTCAGGTTAAGTC	1048
YO11	CAAAATTACAAGGTGCTGCATGGTTGTCGTCAGCTCGTGCCGTGAGGTGTCAGGTTAAGTC	1050
YO15	CAAAATTACAAGGTGCTGCATGGTTGTCGTCAGCTCGTGCCGTGAGGTGTCAGGTTAAGTC	1042
YO19	CAATTTACAAGGTGCTGCATGGTTGTCGTCAGCTCGTGCCGTGAGGTGTCAGGTTAAGTC	1038
YO34	CAATTTACAAGGTGCTGCATGGTTGTCGTCAGCTCGTGCCGTGAGGTGTCAGGTTAAGTC	1043
YO46	CAAAATTACAAGGTGCTGCATGGTTGTCGTCAGCTCGTGCCGTGAGGTGTCAGGTTAAGTC	1039
YO51	CAATTTACAAGGTGCTGCATGGTTGTCGTCAGCTCGTGCCGTGAGGTGTCAGGTTAAGTC	1046
YO60	CAATTTACAAGGTGCTGCATGGTTGTCGTCAGCTCGTGCCGTGAGGTGTCAGGTTAAGTC	1037
YO64	CAATTTACAAGGTGCTGCATGGTTGTCGTCAGCTCGTGCCGTGAGGTGTCAGGTTAAGTC	1021
FJOHNSONIAE	CAATTTACAAGGTGCTGCATGGTTGTCGTCAGCTCGTGCCGTGAGGTGTCAGGTTAAGTC	1076
FRAINBOWTROU	CAATTTACAAGGTGCTGCATGGTTGTCGTCAGCTCGTGCCGTGAGGTGTCAGGTTAAGTC	1068
FWUBA46	CAATTTACAAGGTGCTGCATGGTTGTCGTCAGCTCGTGCCGTGAGGTGTCAGGTTAAGTC	1044
YO10	CTATAACGAGCGCAACCCCTGTTGTTAGTTGCCAGCGAGTCAAGTCGGGAACCTAACAAC	1108
YO11	CTATAACGAGCGCAACCCCTGTTGTTAGTTGCCAGCGAGTCAAGTCGGGAACCTAACAAC	1110
YO15	CTATAACGAGCGCAACCCCTGTTGTTAGTTGCCAGCGAGTCANGTCGGGAACCTAACAAC	1102
YO19	CTATAACGAGCGCAACCCCTGTTGTTAGTTGCCAGCAAGTCAAGTCGGGAACCTAACAAC	1098
YO34	CTATAACGAGCGCAACCCCTGTTGTTAGTTGCCANCGAGTCAAGTCGGGAACCTAACAAC	1103
YO46	CTATAACGAGCGCAACCCCTGTTGTTAGTTGCCAGCGAGTCATGTCGGGAACCTAACAAC	1099
YO51	CTATAACGAGCGCAACCCCTGTTGTTAGTTGCCAGCGAGTCAAGTCGGGAACCTAACAAC	1106
YO60	CTATAACGAGCGCAACCCCTGTTGTTAGTTGCCAGCGAGTCATGTCGGGAACCTAACAAC	1097
YO64	CTATAACGAGCGCAACCCCTGTTGTTAGTTGCCAGCGAGTCAAGTCGGGAACCTAACAAC	1081
FJOHNSONIAE	CTATAACGAGCGCAACCCCTGTTGTTAGTTGCCAGCGAGTCATGTCGGGAACCTAACAAC	1136
FRAINBOWTROU	CTATAACGAGCGCAACCCCTGTTGTTAGTTGCCAGCGAGTCATGTCGGGAACCTAACAAC	1128
FWUBA46	CTATAACGAGCGCAACCCCTGTTGTTAGTTGCCAGCGAGTCATGTCGGGAACCTAACAAC	1104
YO10	GACTGCCAGTGCAAACTGTGAGGAAGGTGGGGATGACGTCAAATCATCACGGCCCTTACG	1168
YO11	GACTGCCAGTGCAAACTGTGAGGAAGGTGGGGATGACGTCAAATCATCACGGCCCTTACG	1170
YO15	GACTGCCAGTGCAAACTGTGAGGAAGGTGGGGATGACGTCAAATCATCACGGCCCTTACG	1162
YO19	GACTGCCAGTGCAAACTGTGAGGAAGGTGGGGATGACGTCAAATCATCACGGCCCTTACG	1158
YO34	GACTGCCAGTGCAAACTGTGAGGAAGGTGGGGATGACGTCAAATCATCACGGCCCTTACG	1163
YO46	GACTGCCAGTGCAAACTGTGAGGAAGGTGGGGATGACGTCAAATCATCACGGCCCTTACG	1159
YO51	GACTGCCAGTGCAAACTGTGAGGAAGGTGGGGATGACGTCAAATCATCACGGCCCTTACG	1166
YO60	GACTGCCAGTGCAAACTGTGAGGAAGGTGGGGATGACGTCAAATCATCACGGCCCTTACG	1157
YO64	GACTGCCAGTGCAAACTGTGAGGAAGGTGGGGATGACGTCAAATCATCACGGCCCTTACG	1141
FJOHNSONIAE	GACTGCCAGTGCAAACTGTGAGGAAGGTGGGGATGACGTCAAATCATCACGGCCCTTACG	1196
FRAINBOWTROU	GACTGCCAGTGCAAACTGTGAGGAAGGTGGGGATGACGTCAAATCATCACGGCCCTTACG	1188
FWUBA46	GACTGCCAGTGCAAACTGTGAGGAAGGTGGGGATGACGTCAAATCATCACGGCCCTTACG	1164

YO10	CCTTGGGCTACACACGTGCTACAATGGCCGGTACAGAGAGCAGCCACTGGGCGACCAGGA	1228
YO11	CCTTGGGCTACACACGTGCTACAATGGCCGGTACAGAGAGCAGCCACTGGGCGACCAGGA	1230
YO15	CCTTGGGCTACACACGTGCTACAATGGCCGGTACAGAGAGCAGCCACTGGGCGACCAGGA	1222
YO19	CCTTGGGCTACACACGTGCTACAATGGCCGGTACAGAGAGCAGCCACTGGGCGACCAGGA	1218
YO34	CCTTGGGCTACACACGTGCTACAATGGCCGGTACAGAGAGCAGCCACTGGGCGACCAGGA	1223
YO46	CCTTGGGCTACACACGTGCTACAATGGCCGGTACAGAGAGCAGCCACTGGGCGACCAGGA	1219
YO51	CCTTGGGCTACACACGTGCTACAATGGCCGGTACAGAGAGCAGCCACTGGGCGACCAGGA	1226
YO60	CCTTGGGCTACACACGTGCTACAATGGCCGGTACAGAGAGCAGCCACTGGGCGACCAGGA	1217
YO64	CCTTGGGCTACACACGTGCTACAATGGCCGGTACAGAGAGCAGCCACTGGGCGACCAGGA	1201
FJOHNSONIAE	CCTTGGGCTACACACGTGCTACAATGGCCGGTACAGAGAGCAGCCACTGGGCGACCAGGA	1256
FRAINBOWTROU	CCTTGGGCTACACACGTGCTACAATGGCCGGTACAGAGAGCAGCCACTGGGCGACCAGGA	1248
FWUBA46	CCTTGGGCTACACACGTGCTACAATGGCCGGTACAGAGAGCAGCCACTGGGCGACCAGGA	1224
YO10	GCGAATCTATAAAACCGGTACAGTTCGGATCGGAGTCTGCAACTCGACTCCGTGAAGCT	1288
YO11	GCGAATCTATAAAACCGGTACAGTTCGGATCGGAGTCTGCAACTCGACTCCGTGAAGCT	1290
YO15	GCGAATCTATAAAACCGGTACAGTTCGGATCGGAGTCTGCAACTCGACTCCGTGAAGCT	1282
YO19	GCGAATCTATAAAACCGGTACAGTTCGGATCGGAGTCTGCAACTCGACTCCGTGAAGCT	1278
YO34	GCGAATCTATAAAACCGGTACAGTTCGGATCGGAGTCTGCAACTCGACTCCGTGAAGCT	1283
YO46	GCGAATCTATAAAACCGGTACAGTTCGGATCGGAGTCTGCAACTCGACTCCGTGAAGCT	1279
YO51	GCGAATCTATAAAACCGGTACAGTTCGGATCGGAGTCTGCAACTCGACTCCGTGAAGCT	1286
YO60	GCGAATCTATAAAACCGGTACAGTTCGGATCGGAGTCTGCAACTCGACTCCGTGAAGCT	1277
YO64	GCGAATCTATAAAACCGGTACAGTTCGGATCGGAGTCTGCAACTCGACTCCGTGAAGCT	1261
FJOHNSONIAE	GCGAATCTATAAAACCGGTACAGTTCGGATCGGAGTCTGCAACTCGACTCCGTGAAGCT	1316
FRAINBOWTROU	GCGAATCTATAAAACCGGTACAGTTCGGATCGGAGTCTGCAACTCGACTCCGTGAAGCT	1308
FWUBA46	GCGAATCTATAAAACCGGTACAGTTCGGATCGGAGTCTGCAACTCGACTCCGTGAAGCT	1284
YO10	GGAATCGCTAGTAATCGGATATCAGCCATGATCCGGTGAATACGTTCCCGGGCCTTGAT	1348
YO11	GGAATCGCTAGTAATCGGATATCAGCCATGATCCGGTGAATACGTTCCCGGGCCTTGAT	1350
YO15	GGAATCGCTAGTAATCGGATATCAGCCATGATCCGGTGAATACGTTCCCGGGCCTTGAT	1342
YO19	GGAATCGCTAGTAATCGGATATCAGCCATGATCCGGTGAATACGTTCCCGGGCCTTGAT	1338
YO34	GGAATCGCTAGTAATCGGATATCAGCCATGATCCGGTGAATACGTTCCCGGGCCTTGAT	1343
YO46	GGAATCGCTAGTAATCGGATATCAGCCATGATCCGGTGAATACGTTCCCGGGCCTTGAT	1339
YO51	GGAATCGCTAGTAATCGGATATCAGCCATGATCCGGTGAATACGTTCCCGGGCCTTGAT	1346
YO60	GGAATCGCTAGTAATCGGATATCAGCCATGATCCGGTGAATACGTTCCCGGGCCTTGAT	1337
YO64	GGAATCGCTAGTAATCGGATATCAGCCATGATCCGGTGAATACGTTCCCGGGCCTTGAT	1321
FJOHNSONIAE	GGAATCGCTAGTAATCGGATATCAGCCATGATCCGGTGAATACGTTCCCGGGCCTTGAT	1376
FRAINBOWTROU	GGAATCGCTAGTAATCGGATATCAGCCATGATCCGGTGAATACGTTCCCGGGCCTTGAT	1368
FWUBA46	GGAATCGCTAGTAATCGGATATCAGCCATGATCCGGTGAATACGTTCCCGGGCCTTGAT	1344
YO10	ACACCGCCCGTCAAGCCATGGAAGCTGGGGGTGCCTGAAGT.CGGTGACCGCAAGGA.GC	1406
YO11	ACACCGCCCGTCAAGCCATGGAAGCTGGGGGTGCCTGAAGT.CGGTGACCGCAAGGA.GC	1408
YO15	ACACCGCCCGTCAAGCCATGGAAGCTGGGGGTGCCTGAAGT.CGGTGACCGCAAGGA.GC	1401
YO19	ACACCGCCCGTCAAGCCATGGAAGCTGGGGGTGCCTGAAGT.CGGTGACCGCAAGGA.GC	1396
YO34	ACACCGCCCGTCAAGCCATGGAAGCTGGGGGTGCCTGAAGT.CGGTGACCGCAAGGA.GC	1403
YO46	ACACCGCCCGTCAAGCCATGGAAGCTGGGGGTGCCTGAAGT.CGGTGACCGCAAGGA.GC	1398
YO51	ACACCGCCCGTCAAGCCATGGAAGCTGGGGGTGCCTGAAGT.CGGTGACCGCAAGGA.GC	1404
YO60	ACACCGCCCGTCAAGCCATGGAAGCTGGGGGTGCCTGAAGT.CGGTGACCGCAAGGA.GC	1396
YO64	ACACCGCCCGTCAAGCCATGGAAGCTGGGGGTGCCTGAAGT.CGGTGACCGCAAGGA.GC	1380
FJOHNSONIAE	ACACCGCCCGTCAAGCCATGGAAGCTGGGGGTGCCTGAAGT.CGGTGACCGCAAGGA.GC	1434
FRAINBOWTROU	ACACCGCCCGTCAAGCCATGGAAGCTGGGGGTGCCTGAAGT.CGGTGACCGCAAGGA.GC	1371
FWUBA46	ACACCGCCCGTCAAGCCATGGAAGCTGGGGGTGCCTGAAGT.CGGTGACCGCAAGGA.GC	1402

YO10	TGCCTA.GGGAAAAAGTGGAAATAGGTG.....	1432
YO11	TGCCTA.GGGAAAACTGGAACTAGGGT.....	1434
YO15	1401
YO19	TTCTTA.GTTAAA.....	1408
YO34	TGCCTATGGGAAAAACTGGAATAG.....	1427
YO46	TGCCTA.GGGAAAACTGGAATAG.....	1421
YO51	TGTTTAAGGGAAAAANCTGGAAATACG.....	1430
YO60	TGCCTA.GGGAAAACTGGAA.....	1416
YO64	TGCCTA.GGGAAAACTGGAAATAG.....	1403
FJOHNSONIAE	TGCCTAGGGTAAAACTGGTAACTAGGGCNNNNNNGTAACAAGNNNNNNNNNNNNNNNNNN	1494
FRAINBOWTROU	1371
FWUBA46	TGCCTA.GGGTAAAACTGGTA.....	1422
YO10	1432
YO11	1434
YO15	1401
YO19	1408
YO34	1427
YO46	1421
YO51	1430
YO60	1416
YO64	1403
FJOHNSONIAE	NNNNNNNGAACACCTCCTTTCT	1516
FRAINBOWTROU	1371
FWUBA46	1422

FIG. 1A. Alignments of 16S rRNA gene sequences of *Flavobacterium* spp. study isolates YO10, YO11, YO15 + YO46 and YO19, YO34, YO64, YO51 + YO60, as well as FJOHNSONIAE AB078043 (Welker *et al.*, 2005), FRAINBOWTROU AY374109 (Huber *et al.*, 2004), and FWUBA46 AF336355 (Bohnert *et al.*, 2001).

APPENDIX 2

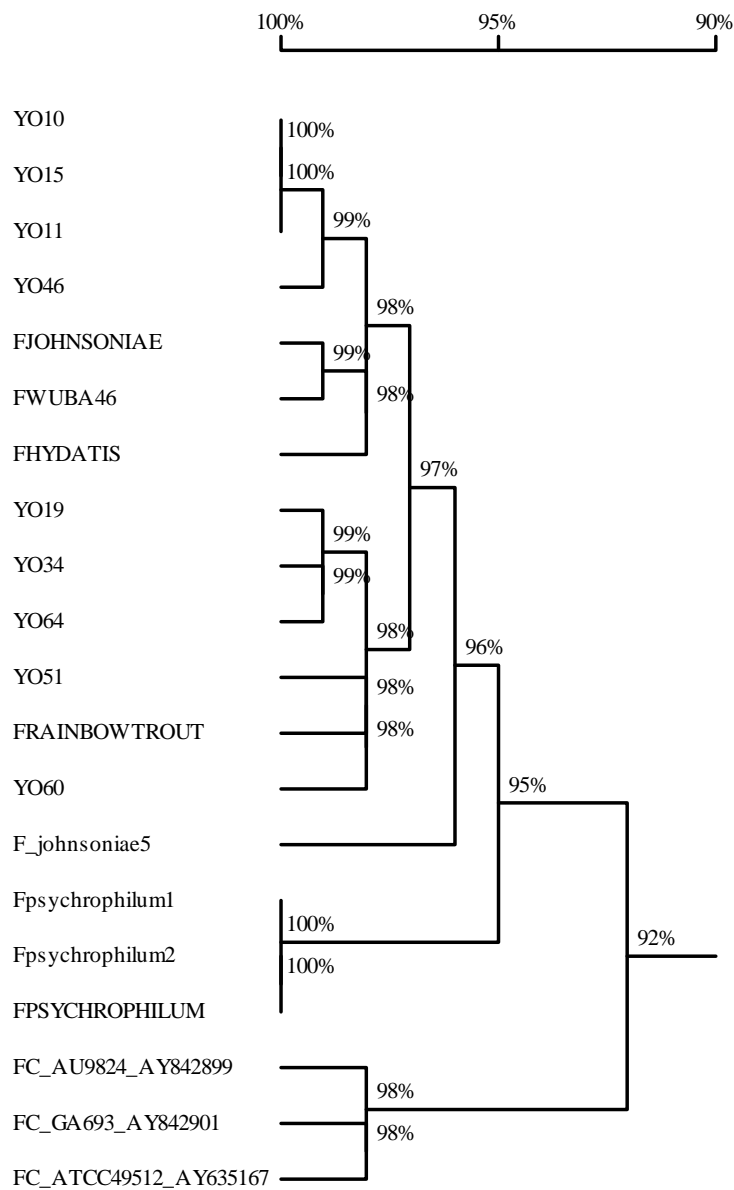


FIG. A. 16S rRNA sequence homology tree showing relatedness of nine selected *Flavobacterium* spp. study isolates (YO10, YO11, YO15, YO19, YO34, YO46, YO51, YO60, and YO64) to other related bacteria in the genus *Flavobacterium*: FC (*F. columnare*) AU9824 AY842899, FC (*F. columnare*) ATCC49512 AY635167 (Darwish and Ismaiel, 2005), FC (*F. columnare*) GA693 AY842901, FHYDATIS AY753068 (Welker *et al.*, 2005), Fjohnsoniae5 AY753067 (Welker *et al.*, 2005), FJOHNSONIAE AB078043 (Nakagawa *et al.*, 2002), FPSYCHROPHILUM AB07860 (Nakagawa *et al.*, 1996), Fpsychrophilum2 AY662493 (Soule *et al.*, 2005), Fpsychrophilum1 AY662494 (Soule *et al.*, 2005), FRAINBOWTROUT AY374109 (Huber *et al.*, 2004), and FWUBA46 AF336355 (Bohnert *et al.*, 2001), based on comparison of 16S rRNA sequences.